

CLONING AND EXPRESSION OF THE ELK (*Cervus elaphus*)
PITUITARY GLYCOPROTEIN HORMONES

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ABSTRACT

The North American elk or wapiti is an indigenous specie to Canada. Understanding of the reproductive physiology of elk is limited, as little research has been conducted in this field as compared to domestic farmed species. In order to make available the tools to study reproductive physiology of the elk this thesis describes the cloning and expression of elk pituitary glycoprotein hormone cDNAs. The common gonadotropin α -subunit, and FSH, LH and TSH β -subunit elk cDNAs were amplified by reverse transcription and polymerase chain reaction (RT-PCR). There was a high degree of nucleotide similarity between the elk α and β subunits when compared with reported sequences from other species. The cDNAs for the pituitary glycoprotein hormone genes were used as probes to investigate seasonal expression in the female elk pituitary gland. Steady state levels of the common α -subunit mRNA was observed regardless of the reproductive season, but a significant increase in expression occurred during the breeding season. The FSH and LH β -subunit genes were expressed at low levels in pituitary glands of animals during presumed anestrus and pregnancy, but levels considerably increased during estrus. In contrast, levels of TSH β -subunit mRNA were similar regardless of the reproductive status. The FSH cDNAs were also transfected into a Chinese hamster ovary (CHO) mammalian expression system, aimed at the production of recombinant elk FSH. Transfected CHO cell lines were screened for expression of α - and FSH β -subunit mRNA by Northern blot. Activity of FSH was equivalent to ~100 mIU/ml of recombinant human FSH (Gonal-FTM), identified by FSH receptor signalling in an *in vitro* cell based assay. In conclusion, this work represents an

advance towards understanding the molecular basis of seasonal reproduction in elk. This information and the availability of elk recombinant FSH will be useful for the application of advanced reproductive technologies required for the rapid expansion of healthy, disease resistant, and genetically superior animals, which are important for domestic production and wildlife management.

DEDICATION

*This thesis is dedicated to my parents Ryan and Roberta Clark,
and my husband Tyrone; for without their constant support
I would not have continued nor completed this program.*

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LIST OF ABBREVIATIONS

| | |
|---------------|--|
| AI | Artificial insemination |
| ATP | Adenosine triphosphate |
| α -MEM | α -minimum essential medium |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| cAMP | 3', 5'-cyclic adenosine monophosphate |
| cDNA | Complementary deoxyribonucleic acid |
| CIDR | Controlled internal drug releasing device |
| CG | Chorionic gonadotropin |
| CHO | Chinese hamster ovary cells |
| CHO-FSH-R | Chinese hamster ovarian cells expressing the human FSH receptor and luciferase genes |
| CMV | Cytomegalovirus |
| CRE | cAMP responsive elements |
| CREB | cAMP response element binding protein |
| CRL-9096 | DHFR- CHO cell line |
| CT | Cholera toxin |
| CWD | Chronic wasting disease |
| DHFR- | Dihydrofolate reductase deficient |
| DMSO | Dimethyl sulfoxide |
| dNTPs | Deoxyribonucleotide triphosphates |

| | |
|----------------|--|
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| eCG | Equine chorionic gonadotropin |
| <i>E.coli</i> | <i>Escherichia coli</i> |
| EDTA | Disodium ethylenediamine-tetra acetate |
| FBS | Fetal bovine serum |
| FSH | Follicle stimulating hormone |
| FSH-R | Follicle stimulating hormone receptor |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GnRH | Gonadotropin releasing hormone |
| hEF-1 α | Human elongation factor 1 α -subunit promoter |
| IBMX | 3-isobutyl-1-methyl-xanthine |
| IPTG | Isopropylthio- β -galactoside |
| IU | International units |
| IVF | <i>In vitro</i> fertilization |
| LB | Luria Bertani |
| LH | Luteinizing hormone |
| MCS | Multiple cloning site |
| M-MLV | Moloney murine leukemia virus |
| MOPS | 3-(N-Morpholino) propanesulfonic acid |
| mRNA | Messenger ribonucleic acid |
| N | Asparagine N-linked glycosylation |
| NCBI | National Centre for Biotechnology Information |

| | |
|------------|--|
| PCR | Polymerase chain reaction |
| PKA | Protein kinase A |
| RER | Rough endoplasmic reticulum |
| RLM-RACE | RNA ligation mediated rapid amplification of cDNA ends |
| RLU | Relative light unit |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcription; polymerase chain reaction |
| Sarkosyl | N-lauroylsarcosine |
| SDS | Sodium dodecyl sulphate |
| TAE | Tris acetate 40 mM, EDTA 2 mM, buffer, pH 8.5 |
| <i>Taq</i> | <i>Thermus aquaticus</i> |
| TB | Tuberculosis |
| TE | Tris-Cl 10 mM, pH 8, EDTA 1 mM, pH 8 buffer |
| TSE | Transmissible spongiform encephalopathy |
| TSH | Thyroid stimulating hormone |
| URE | Upstream regulatory elements |
| UTR | Untranslated region |
| X-gal | 5-bromo-4-choro-3-indolyl-B-D-galactoside |

1. GENERAL INTRODUCTION

The North American elk (*Cervus elaphus*) or wapiti is indigenous to Canada (Toweill and Thomas, 2002). Elk are closely related to European red deer (*Cervus elaphus*). Elk colonized North America from Russia by crossing the Bering Strait (Guthrie, 1966; Murie, 1979). From the late 1970's to the mid 1990's farmed elk became a viable agricultural livestock alternative to traditional cattle/grain farming practices in Canada (Thorleifson et al., 1997). Elk offer many farming advantages including: 1) Efficiency in land use compared to cattle, as four lactating elk cows can be fed on the same pasture that would support only one beef cow; 2) Conservation of native North American grassland, because elk prefer browsing to grazing, thereby making efficient utilization of marginal land; 3) The relative ease of calving, since parturition in elk occurs mostly during the day and does not require human assistance; 4) Elk herd behaviour ensures protection of calves from predators; and 5) As a native specie elk are well suited to the extremes of Canadian weather (Thorleifson et al., 1997). Thus, elk represent an attractive alternative to the traditional livestock industry.

Elk are seasonal breeders and reproductive activity coincides with decreasing daylight hours (Asher et al., 2000a and 2000b). Calves are born when new spring vegetative growth supports the heavy nutritional requirements of lactation (Haigh, 2001). Many other physiological processes such as metabolism, growth and shedding of the male's antlers are also under seasonal control (Suttie et al., 1992). Knowledge of elk reproductive physiology is limited, as little research has been conducted in this field

as compared to traditional domestic farmed species. Therefore, there is a need for more research on the reproductive biology of elk to improve their reproductive efficiency and to promote production of healthy and genetically superior animals.

1.1 *Economical significance of elk*

Cervids have been farmed in countries, such as China and Siberia, according to written records, dating back some 2500 years (Wall and Knopf, 1993). Traditionally Asian cultures have utilized cervid antler products as additives in medicinal formulas to improve strength, energy and good health (Wall and Knopf, 1993). Elk represent a commodity of great economic potential for Canadian farmers. The sale and export of elk velvet, harvested from the males every spring has the potential to be a lucrative business. The elk velvet produced in Canada has been of high quality and represents a commodity sought by many Asian countries. In addition, the potential of elk as a food source is also recognized, as venison is regarded as a lean, healthy alternative to beef. The market potential for elk venison has not been developed, mainly due to the lack of sufficient numbers to supply the demand.

The importance of elk in Western Canadian economy is now underscored by the loss caused by chronic wasting disease (CWD). Chronic wasting disease belongs to the family of diseases termed transmissible spongiform encephalopathies (TSE), caused by an abnormally folded endogenous cellular protein, termed a prion. Prion diseases are incurable and result in death (Williams and Young, 1993). The post-mortem morphology includes degeneration of the central nervous system tissue and neuronal vacuolation, giving the brain a spongiform appearance (Prusiner et al., 1998). It is believed that CWD was first characterized in a captive mule deer at the Foothills

Wildlife Research Station near Fort Collins, Colorado, USA. The captive mule deer exhibited strange behaviour and died in 1967 (Dunphy, 2001). In Canada, CWD was diagnosed at an elk farm in Saskatchewan in 1996. Since then, more farms have been infected and thousands of animals have been slaughtered (Dunphy, 2001; Williams and Young, 1980; and Spraker et al., 1997) as part of a federally regulated control program.

Alternatives to controlling the disease include selective breeding for genetic resistance to the prion disease, which has been effective as a means of controlling the prion disease, scrapie, in sheep (Dawson et al., 1998). In elk, genetic polymorphisms within the prion gene have been discovered and have been correlated with the occurrence of CWD. Identification of elk carrying specific alleles may become an attractive avenue to control the disease (O'Rourke, et al., 1999; Raymond, et al., 2000). Thus, we foresee the need for putting into practice modern assisted reproductive technologies, including artificial insemination, embryo transfer and *in vitro* fertilization (IVF), for the rapid re-establishment of healthy CWD disease resistant herds of elk.

1.2 *The glycoprotein hormone family*

The pituitary glycoprotein hormone family includes the gonadotropins FSH and LH, which are produced by the gonadotrope cells, and the structurally related TSH, produced by the thyrotope cells of the anterior pituitary gland. In addition, chorionic gonadotropins are synthesized by the placental trophoblast cells of primates (hCG) and equids (eCG). These glycoproteins are heterodimers, composed of a common α -subunit and a hormone-specific β -subunit. The α - and β - subunits are non-covalently linked and have carbohydrate side chains attached to each of the subunits. These carbohydrates

play a crucial role in regulating the biological activity of the hormones and their half-life in circulation (Bishop et al., 1994). Carbohydrates are added to FSH, LH and TSH by post-translational N-linked glycosylation and represent around 20% of the mass of the hormone (Huhtaniemi and Aittomaki, 1998). In addition to the N-glycosylated residues, CG has multiple O-glycosylated sugar residues attached to its β -subunit C-terminus, which makes it the longest-lived gonadotropin in circulation.

Post-translational glycosylation of the gonadotropin subunits begins in the rough endoplasmic reticulum (RER) with the co-translational transfer of a dolichol-linked $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharide precursor to the nitrogen amide of asparagine at glycosylation consensus sites Asn-X-Ser/Thr (Baenziger and Green, 1988). This process is not uniform and renders a family of heterogeneous molecules, which circulates as a collection of different carbohydrate structures, including neutral, sialylated, sulfated or mixed sialylated/sulfated isoforms. The isoform hormone profile is different depending on the hormone, species and can shift with age, season or reproductive status (Ulloa-Aguirre et al., 1988). Terminal sulfation favours the removal of the gonadotropin from circulation by the action of a hepatic reticuloendothelial cell binding protein for sulfated glycoproteins (Fiete et al., 1991). Sialic acid decreases hepatic uptake and consequently decreases the degradation rate of the molecule (Ulloa-Aguirre et al., 2003). The crystal structure of deglycosylated hCG has been deciphered and is likely to be representative of each of the glycoprotein hormones (Lapthorn et al., 1994). There are 5 disulphide bonds in the α -subunit and 6 in the β -subunit that form the secondary structure of each subunit. The pairing of cysteine residues creating the disulphide bonds has been extensively studied and debated. The most reliable

assignment includes intra-chain disulphide bonds between cysteines 7-31, 10-60, 28-82, 32-84, and 59-87 for the α -subunit, and the intra-chain disulfide bonds between residues 9-57, 23-72, 26-110, 34-90, and 93-100 for the β -subunit (Lapthorn et al., 1994). Both subunits are composed of three large loops held together by a cysteine knot (Fox et al., 2001), which is similar to some of the protein growth factors, such as transforming growth factor-beta (Vitt et al., 2001). The β -subunit residues 91-110 form an additional loop termed the “seat-belt” that wraps around the second loop of the α -subunit to stabilize the heterodimer (Lapthorn et al., 1994).

The organization of the common glycoprotein α -subunit gene includes four exons separated by three introns (Goodwin et al., 1983). In the pituitary, two cell types, the gonadotropes and thyrotropes express the α -subunit. In primates and equids the α -subunit is expressed in the placental trophoblast cells. The upstream region of the human α -subunit gene contains two 18 base pair cAMP responsive elements (CRE), in addition to a single upstream regulatory element (URE) (Jameson et al., 1989) which are required for placental expression. The α -subunit from other species that do not produce placental gonadotropin, have only one CRE (Nilson et al., 1991).

The organizations of the β -subunit genes FSH, LH and TSH are all the same, having 3 exons separated by two introns. The FSH β -subunit mRNA is unique as it has a relatively long 3' untranslated region compared to the other glycoprotein subunit mRNA structures. The 3' UTR of FSH β -subunit mRNA contains numerous copies of AUUUA, a sequence motif, which has been shown to regulate mRNA stability of other transcripts (Wilson and Treisman, 1988). The gene structure of LH and CG are related since the genes arose from the modification of a duplicated gene copy (Hollenberg et al.,

1994). A single base pair change which eliminated the LH β -subunit stop codon allows the read through into the 3' untranslated region and resulted in an additional 16 residues, the second base pair change was the insertion of two bases that continues the open reading frame to code for an additional 8 residues to produce the hormone CG (Ward et al., 1991).

1.3 *Cognate glycoprotein receptors*

Follicle stimulating hormone receptors (FSH-R) are expressed in the ovarian granulosa cells and in the testicular Sertoli cells (Simoni et al., 1997). The LH receptors (LH-R) are primarily expressed in the ovarian thecal cells, in the granulosa cells of pre-ovulatory follicles and in the testicular Leydig cells (McDonald and Pineda, 1989; Allan et al., 2001). Both CG and LH cross-react with the LH-R, since these hormones share many identical amino acids, which confer similar affinity (Metsikko et al., 1990). The TSH receptors are expressed in the follicular cells of the thyroid gland (Kakinuma and Nagayama, 2002). These receptors are a subclass of the G-protein associated receptor family, which span the plasma membrane seven times. The glycoprotein hormones act on their respective specific receptors, stimulating the synthesis of the second messenger 3', 5'-cyclic adenosine monophosphate (cAMP) and activating the protein kinase-A (PKA) signalling pathway. Activation of the PKA pathway results in the phosphorylation of intracellular kinases and activation of transcription factors, including cAMP response element binding protein (CREB), which binds to the cAMP responsive elements (CRE) located in the promoter regions of a variety of responsive genes (Simoni et al., 1997). Recent information suggests that in addition to binding to its seven

transmembrane receptor, termed FSH-R1, FSH also binds to a single membrane-spanning splice variant of the FSH-R gene, termed FSH-R3 (Babu et al., 1999). Follicle stimulating hormone, by binding to the FSH-R1 stimulates steroid synthesis; while by binding to the FSH-R3, stimulates granulosa cell proliferation (Babu et al., 2000).

Specific bioassays for the glycoprotein hormones have been developed by stable transfection and expression of their respective receptor genes in Chinese hamster ovary (CHO) cells; which are of epithelial origin and do not possess endogenous FSH receptors. The CHO cells were double-transformed with the FSH-R1 and a genomic plasmid construct containing the gonadotropin α -subunit promoter region linked to the luciferase gene (Albanese et al., 1994; Braileanu et al., 1998). The α -subunit promoter region contains two CRE, which respond to cAMP stimulated by FSH signal transduction through the FSH-R1. Therefore, these transformed CHO cells, which respond specifically to FSH with dose-dependent increases in luciferase activity are used in bioassays. Luciferase activity is easily quantified by a luminometric assay, resulting in a valuable tool to test the bioactivity of natural and recombinant preparations of FSH (Albanese et al., 1994; Braileanu et al., 1998). Other FSH bioassays exist, including measurement of estradiol accumulation in porcine granulosa cells, a reflection of FSH-induced aromatase activity (Sites et al., 1996), aromatase activity in rat Sertoli cells (Simoni et al., 1994) and increases in plasminogen activator measured in rat granulosa cells (Thakur et al., 1990). The cell line developed by Albanese et al., 1994 has advantages over these other FSH bioassays in that there is no requirement for primary cell cultures, which are labour intensive, the time required for the assay is reduced from upwards to 5 days to only 2 days and there is not the need for radioisotope use.

1.4 *Production of recombinant gonadotropins*

Recombinant glycoproteins have been produced in a variety of host systems. The ideal host system will produce a properly folded protein with appropriate post-translational modifications, such as a glycosylation pattern resembling the natural hormone, which ensures adequate *in vivo* biological activity. Other considerations include the use of a gene delivery system containing an appropriate selectable marker for rapid identification and amplification of the desired clones, and an efficient method of product purification.

A tobacco mosaic virus based transient expression system was used to express bovine FSH, as a single chain protein, in the tobacco related species *Nicotiana benthamiana*, which produced high levels of recombinant FSH, up to 3% of total soluble protein (Dirnberger et al., 2001). Plants do not express glycosylation enzymes similar to mammals therefore, the recombinant FSH produced by plant cells did not possess terminal sialic acid residues. Thus, this procedure resulted in a product with much lower *in vivo* activity, due to its short half-life in circulation. The methylotrophic yeast, *Pichia pastoris* has also been used to produce recombinant FSH. This system yielded 10 mg/l of FSH, the highest level of production recorded (Richard et al., 1998). However, the yeast produced a recombinant FSH that bears only high-mannose N-linked carbohydrates, with lower heterogeneity as compared to those produced by the pituitary gland, and also lacks terminal sialic acid residues, resulting in low *in vivo* activity. Recombinant porcine FSH has been produced in an insect cell system infected with a baculovirus vector carrying the FSH genes, which produced relatively high levels of

FSH (1 mg/l) of high biological activity (Kato et al., 1998). These insect cell systems are able to carry out N-glycosylation by adding a high molecular mass mannose-type polysaccharide, but have limited ability to trim the terminal sugars, rendering a product that does not bear terminal sialic acid to resemble pituitary produced FSH. Recombinant pituitary glycoproteins have also been produced in the embryonic kidney cell line, HEK-293 (Flack et al., 1994). This method produced basic isoforms of FSH, lacking terminal sialic acid, of high *in vitro* activity but short *in vivo* half-life.

Recombinant cynomolgus monkey LH and FSH has been produced in COS7 and CHO cells which resulted in the secretion of 20 IU/L of *in vitro* bioactive hormones (Schmidt et al., 1999). Recombinant hCG has been produced in CHO cells, which were capable of producing the hormone at the rate of 2-5 µg/ml per 10⁶ cells per 24 hours (Keene et al., 1989). Recombinant rat FSH was produced in CHO cells (Hakola et al., 1997). This product had a pI distribution similar to rat pituitary FSH as determined by isoelectric focusing in immobilized pH gradients (Hakola et al., 1997). Rat recombinant FSH displayed dose-response curves parallel and in the same dose range as the rat pituitary FSH in receptor binding assays and *in vitro* bioassays. The *in vivo* activities of rat recombinant FSH and rat pituitary FSH were 8824 and 3051 IU/mg, respectively determined by the assay described by Steelman and Pohley (1953). Recombinant human FSH has been produced in CHO cells (Olijve et al., 1996). This product had an isoform distribution, similar to pituitary and urinary FSH. Furthermore the carbohydrates showed a pattern of variation in their degree of sialylation, which reflects the different pI, similar to the pituitary FSH (Olijve et al., 1996). Overall, this information suggests that plant, yeast and insect expression systems produce mass quantities of glycoproteins

of limited *in vivo* biological activity. Whereas, the mammalian expression systems, including CHO cells, although less efficient, produce glycoproteins that are biochemically, immunologically and biologically more similar to their natural counterpart.

1.5 *The use of gonadotropins in assisted reproductive technologies*

Presently, the assisted reproductive technology that has the widest use to disseminate genetics from superior cervids is artificial insemination with fresh or frozen semen collected by electro ejaculation. Fixed-time artificial insemination is preferred since natural heat is difficult to detect in red deer (Asher et al, 1993). Estrus is synchronized in hinds with progesterone-impregnated intravaginal controlled internal drug-releasing (CIDR) devices for 12-14 days (device replacement on day 8). Eight equal intramuscular injections of ovine FSH, total of 0.4 IU Ovagen, at 12 hour intervals is initiated at CIDR device replacement, 200 IU eCG Folligon, is incorporated in the final injection. Hinds are inseminated at 44 and 68 hours after CIDR removal with 25 X 10⁶ deer spermatozoa, achieving 40-60% conception rates (Fennessy et al., 1990; Asher et al., 1993). The problem with this procedure is that although most of the large follicles ovulated during this period, nearly 40% persisted, many of which became abnormally large (>15 mm diameter) (Asher et al., 1997). Thus, treatment with eCG induces a high proportion of large follicles that fail to rupture, and ovine FSH alone results in a proportion of animals that fail to respond to treatment (Bainbridge et al., 1996; Asher et al., 1997), this necessitates the need to research alternative products to consistently achieve acceptable conception rates.

Other assisted reproductive technologies such as embryo transfer and *in vitro* fertilization rely on the induction of multiple ovulations, using a pharmacological agent, to obtain multiple ova from a single monovulatory animal, such as elk (Ginther et al., 2001). This procedure, called superovulation, is based on treatments with exogenous gonadotropins, such as follicle-stimulating hormone (FSH) or equine chorionic gonadotropin (eCG). Follicle stimulating hormone and eCG stimulate follicular growth and/or prevent atresia of other follicles in the group, causing them to mature and ovulate in some species at the same time. Although treatment with FSH is sufficient to cause ovulation in some species, such as cattle, other species, such as human, pig, sheep, and probably elk, may require a subsequent treatment with LH to induce follicular rupture. Then, oocytes can be recovered by aspiration, to be used for IVF. In addition, after natural or artificial insemination of a superovulated animal, multiple embryos can be collected, by uterine flushing, for embryo transfer to surrogate recipients.

Although the methodology for ovarian stimulation in domestic animals has been available for many years, the ovarian responses in terms of viable eggs are still unpredictable (Armstrong, 1993). Thus, superovulation is the main factor limiting the success of an assisted reproductive technology program for all species. The gonadotropins presently used for animal superovulation are extracted from domestic animal pituitary glands, which are collected at slaughter and pooled, regardless of sex, age, or species. Since the material is from animals of differing reproductive status, the resulting preparations yield a biochemically undefined product with high variability in FSH and LH activity (Braileanu et al., 1998). Overall, it is believed that the quality of the hormone preparations used is one of the main causes of the unpredictable responses to superovulatory treatments (Armstrong, 1993).

In elk, the problems associated with successful superovulation are even more complicated. The protocols and hormones used have been designed for other species and limited research has been conducted in elk (Pollard and Plante, 2001). Thus, there is a need for better superovulatory agents and protocols specifically designed for elk. To be able to produce natural elk FSH, pituitary glands would need to be collected from a large number of animals. This is not an option, since the numbers of healthy animals to be slaughtered are insufficient. With the advent of molecular biology, it has become possible to clone the gonadotropin genes from a variety of species and to produce species-specific gonadotropins by recombinant methods. Recombinant hormones are very pure, uniform and equally or more effective than natural preparations (Gonzalez et al., 2001). In addition, since they are produced *in vitro* under completely controlled conditions, the risk of disease transmission is practically eliminated. This is very important in elk due to the possibility of CWD transmission through pituitary tissues. Overall, the availability of recombinant gonadotropins would be of significant biotechnological advance for the development of superovulatory protocols specifically designed for elk.

2. OBJECTIVES

- To clone and express the elk gonadotropin cDNAs in a mammalian expression system;
- To investigate the seasonal expression of the gonadotropin genes in the female elk pituitary gland;
- To produce biologically active recombinant elk FSH.

3. GENERAL MATERIALS AND METHODS

3.1 Reagents and Materials

General laboratory reagents, TRI[®] Reagent, Cholera toxin (CT) and luciferin, were purchased from Sigma (Oakville, ON). Oligotex, pDRIVE polymerase chain reaction (PCR) cloning, QiaexII, Qiagen[®] plasmid DNA purification columns and QIAquick nucleotide removal kits, were purchased from Qiagen (Mississauga, ON). Moloney Murine Leukemia virus (M-MLV) reverse transcriptase, *Thermus aquaticus* (Taq) polymerase, restriction endonuclease enzymes, oligonucleotide custom primers, dNTPs, MgCl₂, 10X PCR buffer, random primers, TOPO TA PCR cloning kit, *Escherichia coli* (*E. coli*) strain TOP 10 competent cells, pBudCE4.1 vector and antibiotic Zeocin[™] were purchased from Invitrogen (Carlsbad, CA). Ampicillin, 5-bromo-4-choro-3-indolyl-B-D-galactoside (X-gal), Isopropylthio-β-galactoside (IPTG), fetal bovine serum (FBS), α-minimum essential medium (α-MEM) powder, gentamicin, Geneticin[®], trypsin, Fungizone[®], Lipofectamine 2000, T4 DNA ligase and fungal Proteinase K were purchased from Gibco BRL (Burlington, ON). Oligo (dT)-Cellulose Type 7, Hybond-N+ nylon membranes and Sephadex G-10 column were purchased from Amersham Biosciences (Piscataway, NJ). First Choice RNA Ligation Mediated-Rapid Amplification of cDNA ends (RLM-RACE) kit was purchased from Ambion (Austin, TX). [α-³²P]-dCTP was purchased from PerkinElmer (Boston, MA). Kodak X-OMAT AR X-ray Scientific Imaging (Eastman Kodak, Rochester, NY). Recombinant human FSH (Gonal-F[™]) was obtained from Serono Canada Inc. (Oakville, ON). Reagents for

bacterial growth, Bacto-Agar, Tryptone-peptone and yeast extract were purchased from Difco Laboratories (Detroit, MI). The dihydrofolate reductase deficient (DHFR) Chinese hamster ovary (CHO) cells, CRL-9096 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Falcon (Lincoln Park, NJ) plastic tissue culture plates.

3.2 Sample collection, RNA isolation and cDNA synthesis

Muscle samples and pituitary glands, from elk, sheep and horses, were removed immediately after slaughter, minced in TRI[®] Reagent, frozen in liquid nitrogen and kept at -80°C until processed for RNA isolation. Blood samples were collected in BD Vacutainer[™] (Franklin Lakes, NJ) test tubes containing anticoagulants and stored at -20°C until processed. Tissue was homogenized with a Polytron[®] tissue homogenizer (Brinkmann, Mississauga, ON) and total RNA was isolated following the manufacture's recommendations for TRI[®] Reagent, or the protocol outlined by Chomczynski and Sacchi (1987). Polyadenylated RNA was isolated from total RNA samples by affinity chromatography using oligo (dT)-cellulose (Appendix 2A), or purified using the Oligotex[®] kit following the manufacture's recommendations. Polyadenylated RNA was reverse transcribed using either a poly (dT) primer and the M-MLV reverse transcriptase or Ambion's RLM-RACE kit following the manufacture's recommendations.

3.3 cDNA amplification, cloning and sequencing

Oligonucleotide primers, listed in Table 1, were designed based on sequences published to the National Center for Biotechnology Information (NCBI) GenBank and custom synthesized by Invitrogen. PCR was conducted using the custom primers and

the reagents provided by the manufacturer. The PCR mixture included 2 μ l of RT-cDNA, 5 μ l of the 10X PCR buffer, 4 μ l of 2.5 mM dNTP, 2.5 μ l of 50 mM MgCl₂, 50 pmol of reverse and forward primers, and 1.5 units of *Taq* DNA polymerase. DNA amplification was performed in a MinicyclerTM (MJ Research Inc., Watertown, MA) with the following cycling conditions: (1) 94°C for 3 min, (2) 94° C for 1 min, (3) 60-65°C for 1 min, (4) 72°C for 1-2 min, (5) 30 cycles of steps 2-4, and 6) a terminal extension step at 72°C for 7 min.

| Primer | Sequence |
|---------------------------------------|--|
| Forward α -subunit | 5' AGGCAGAGGACGAAGAGCCATGGA 3' Designed based on ovine NCBI GenBank Accession number X16977, nucleotides 52-75. Two nucleotides were changed to deter primer dimmer formation. |
| Reverse 3' RACE inner | 5' CGCGGATCCGAATTAATACGACTCACTATAGG 3' From the Ambion RLM-RACE kit Expected PCR product of 695 bp |
| Forward FSH β -subunit | 5' AGCATCCACAGTTACCAAGTGC 3' Designed based on bovine NCBI GenBank Accession number M14853.1, nucleotides 20-41. Two nucleotides were changed to deter primer dimmer formation. |
| Reverse FSH β -subunit primer 1 | 5' CTGAAGGAGCAGTAGCTGGGC 3' Designed based on ovine NCBI GenBank Accession number X15493, nucleotides 400-420. Expect PCR product of 400 bp |
| Genomic Forward FSH β -subunit | 5' AGCAGTATTCAATCCCTGTCTCA 3' Designed based on ovine NCBI GenBank Accession number S64745, nucleotides 2338-2360. |
| Genomic Reverse FSH β -subunit | 5' CATGTACACACAGACAGCTTGGATG 3' Designed based on ovine NCBI GenBank Accession number S64745, nucleotides 2736-2760. 1 mismatch Expect PCR product of 422 bp |
| Forward FSH β -subunit | 5' AGCATCCACAGTTACCAAGTGC 3' Designed based on ovine NCBI GenBank Accession number X15493, nucleotides 20-41. |
| Reverse FSH β -subunit primer 2 | 5' TTA CTCTCTGACGTCGCTGAAGGAGC 3' Designed based on genomic elk FSH β -subunit PCR product. Expect PCR product of 414 bp |

| | |
|----------------------------------|---|
| Forward LH β-subunit | 5' GGTACCAGGCACCAAGGATGGAG 3' Designed based on ovine NCBI GenBank Accession number S64695, nucleotides 707-723, 6 nucleotides added to the 5' end for a <i>Kpn</i> I restriction endonuclease site. |
| Reverse LH β-subunit | 5' AGAAGTCTTTATTGGGAAGGGAGG 3' Designed based on ovine NCBI GenBank Accession number S64695, nucleotides 1748-1771. Expect PCR product of 525 bp |
| Forward TSH β-subunit | 5' AGAGCTTTT AGTCTGGGTCATTACAA 3' Designed based on ovine NCBI GenBank Accession number X90775, nucleotides 1-26. |
| Reverse TSH β-subunit | 5' CTATTAAAATTAGATAGAAAATCCC 3' Designed based on ovine NCBI GenBank Accession number X90775, nucleotides 454-478. Expect PCR product of 478 bp |
| Forward FSH β-subunit | 5' AGCATCCACAGTTACCAAGTGC 3' Designed based on ovine NCBI GenBank Accession number X15493, nucleotides 20-41. |
| Reverse FSH Th1 | 5' TCGAGGCACCAGCTCTCTGACGTCGCTGAAG 3' |
| Reverse Thrombin <i>Xho</i> I | 5' CTCGAGATGCTTCCTCGTGGCACCAG 3' |

Table 1. List of oligonucleotide primers.

The PCR products amplified were separated by electrophoresis through a 1% agarose gel in 1X TAE buffer. The DNA fragments of expected size were excised from the gel with a scalpel and purified using the QiaexII kit, following manufacture's instructions. Ligation was performed in a mixture containing 100 ng of vector DNA plus the insert DNA in 10-fold excess, and incubated with 1 unit of T4 DNA ligase and appropriate buffer at 14°C overnight. A 5 µl aliquot of the ligation mixture was used to transform 100 µl of competent *E.coli* cells. Transformed cells were spread onto a plate containing Luria Bertani (LB) broth, agar and 50 µg/ml of the appropriate antibiotic, either ampicillin or ZeocinTM. Plasmid DNA was isolated with Qiagen[®] plasmid DNA columns and sequenced using forward and reverse universal primers or custom designed primers with a fluorescent automated sequencer (National Research Council, Plant Biotechnology Institute, Saskatoon, SK).

3.4 DNA isolation from whole blood

Blood samples were collected from the animal into VacutainerTM tubes and frozen at -20°C until processed. Blood samples were thawed in ice water and genomic DNA extracted by triton/sucrose lysis procedure as it is described in Appendix 2B.

3.5 Northern blot analyses

RNA samples were size fractionated by electrophoresis through a 1% agarose/formaldehyde gel in 1X 3-(N-Morpholino) propanesulfonic acid (MOPS) buffer and transferred to a Hybond-H⁺ nylon membrane by capillary blotting in 20X SSC buffer (Appendix 1) using a Schleicher and Schuell TurboblotterTM (Keene, NH). RNA was then fixed to the membrane by UV-cross-linking using a Stratalinker 1800[®]

(Stratagene, La Jolla, CA). Membranes were soaked in water, transferred to a hybridization bottle, pre-hybridized for 2 hours (Appendix 1). Probes were labelled by primer extension with 50 μCi [α - ^{32}P]-dCTP, purified by a QIAquick[®] nucleotide removal column following the manufacture's recommendations, then mixed with 100 μl of salmon sperm DNA and 100 μl of 1M NaCl, boiled for 5 minutes and added directly to the hybridization solution. Membranes were hybridized by overnight incubation at 60-65°C in a model 2000 Micro Hybridization incubator (Robbins Scientific Corporation, Sunnyvale, CA). Membranes were then washed three times with 2X SSC and 0.1% SDS at room temperature for 15 minutes, once with 0.1X SSC and 0.1% SDS at 60-65°C for 45 minutes and a final wash with 2X SSC and 0.1% SDS at room temperature for 15 minutes. Membranes were then exposed to Kodak X-OMAT film, which was developed in an automatic film developer. Mean band intensity in the autoradiograms was determined using the Kodak Digital Science[™] 1D Image Analysis Software System 120 (Eastman Kodak Company, Rochester, NY).

3.6 Vector construction

A DNA fragment of ~720 bp in length, was amplified by RT-PCR with the custom forward primer, termed Forward α -subunit and the 3' RACE inner reverse primer from the Ambion FirstChoice[™] RLM-RACE kit, which included the recognition sequence for the restriction enzyme *Bam*HI. This fragment was cloned into the TOPO TA vector and identified as the elk gonadotropin α -subunit cDNA; it included its endogenous stop codon and polyadenylation signal sequence. For mammalian expression purposes, the elk gonadotropin α -subunit cDNA was sub-cloned into the *Bam*HI cloning site located downstream of the Cytomegalovirus (CMV) promoter of the

pBudCE4.1 vector, shown in Figure 1. This construct was termed pBudCE4.1- α -subunit.

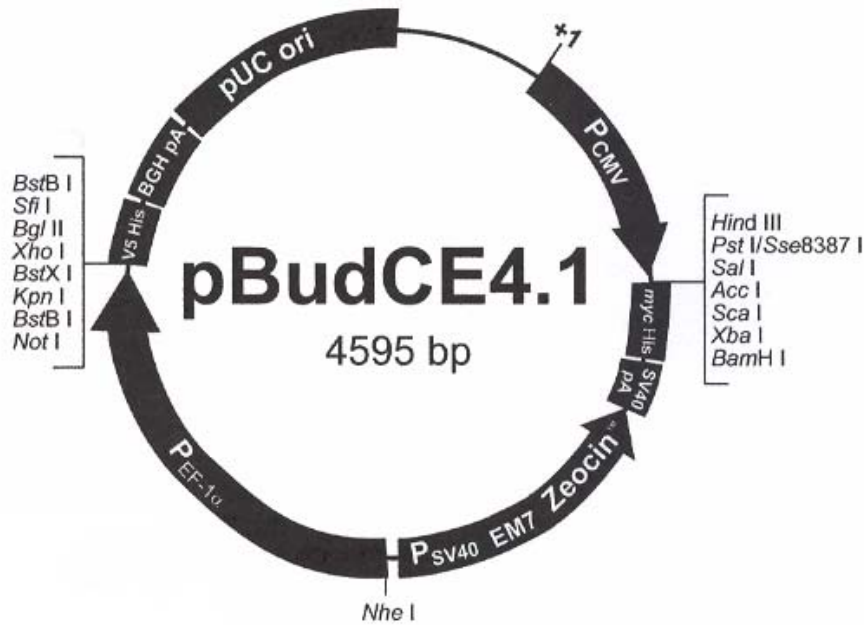


Figure 1. The mammalian dual expression vector pBudCE4.1, illustration adapted from Invitrogen Catalog no. V532-20. This mammalian expression vector has two multiple cloning sites, designed for simultaneous expression of two genes. This vector carries the ZeocinTM resistance gene for selection in *E.coli* and creation of stable mammalian cell lines, the pUC origin of replication (ori) for replication in bacterial cells and the human cytomegalovirus (CMV) immediate-early promoter and the human elongation factor 1 α -subunit (hEF-1 α) promoter for high-level, constitutive, independent expression of two recombinant proteins. The option exists to engineer a C-terminal peptide extension encoding either the *c-myc* or the V5 epitope and a polyhistidine (6Xhis) metal-binding tag for detection and purification of recombinant proteins.

The FSH β -subunit was amplified by RT-PCR using the custom designed primers termed Forward FSH β -subunit primer and Reverse FSH β -subunit primer 2. A DNA fragment of ~500 bp in length was isolated and cloned into the pDRIVE vector. This fragment was identified as the elk FSH β -subunit cDNA by sequencing analysis, which did not include the 3' UTR. The first cloning strategy involved sub-cloning the elk FSH β -subunit cDNA into the *EcoRI* site of the TOPO TA vector, gaining the *KpnI* / *XhoI* restriction sites that were used for cloning into the pBudCE4.1-elk- α -subunit vector. Thus, the elk FSH β -subunit cDNA become under the control of the human elongation factor 1 α (hEF-1 α) promoter and upstream of the bovine growth hormone polyadenylation signal of the pBudCE4.1 (Figure 8). This construct was termed pBudCE4.1-elk- α - β -subunit.

The second cloning strategy was devised to take advantage of the V5 epitope and the 6 consecutive histidines included in the backbone sequence of the pBudCE4.1 vector (Figure 1). For this purpose, the 3' end of the elk FSH β -subunit cDNA was engineered by PCR, using the reverse primers termed WFSHBth1 and Thrombin *XhoI*. In the resulting construct, the endogenous stop codon was deleted, whereas codons encoding the thrombin recognition sequence (Leu Val Pro Arg/Gly Ser) and an in-frame *XhoI* restriction site were added. Thus, the new vector would express a histidine tag, directed to facilitate subsequent recombinant protein purification, and the thrombin recognition site could be used to digest the engineered C-terminal amino acids after purification. The resulting clones were confirmed by sequencing analysis. This engineered elk FSH β -subunit cDNA was cloned into the pBudCE4.1-elk- α -subunit vector using the same

cloning strategy as described for the cloning the elk FSH β -subunit cDNA. This vector was termed pBudCE4.1-elk- α - β -His.

3.7 Transfection and selection of stable CHO cells

CHO cells were cultured in α -MEM media with 10% FBS, and 50 μ g/ml gentamicin (culture media, Appendix 1) at 37°C in a water humidified atmosphere containing 5% CO₂. Once cells reached confluence they were washed with sterile saline, removed by incubation with 1X trypsin and plated at 30% confluence in 35 mm plates in 2 ml of pre-transfection media (Appendix 1). Cells were then transfected with the addition of 500 μ l of transfection media containing 4 μ g of plasmid DNA and 8 μ l of Lipofectamine 2000 per well for 24 h. Cells were then removed, sub-cultured at a dilution of 1:12. After 24 hours of incubation, media was then replaced with selection media containing 500 μ g/ml of ZeocinTM. Media was changed every 3 days, for 7-10 days, until colonies were visualized. Single colonies were then isolated with a sterile filter paper disc soaked in trypsin and transferred to separate wells for further culturing (Domann and Martinez, 1995). Samples from each colony were collected for RNA extraction with TRI[®] Reagent for Northern blot analysis. Frozen samples of each cell line were stored in liquid nitrogen in a solution containing FBS with 10% dimethyl sulfoxide (DMSO) for long-term storage.

3.8 FSH bioassay

For testing FSH bioactivity, transformed cells were cultured to confluency. Then, culture media was replaced by 6 ml of α -MEM media supplemented with gentamicin (collection media, Appendix 1). After 6 days of incubation, collection media

from each colony was aspirated and centrifuged at 300 rpm for 10 min to pellet cell debris. The cleared collection media was stored at -20°C until assayed for FSH bioactivity.

Bioactivity was tested using the assay described by Albanese et al. (1994). This assay is based on a CHO cell line, termed CHO-FSH-R, that stably express the human FSH receptor and a cAMP responsive promoter driving the expression of the reporter gene luciferase which were received as a gift from Northwestern University Medical School, Chicago, IL. CHO-FSH-R cells were maintained in culture media containing 200 µg/ml of Geneticin[®] which is the selection agent for transformed cells. For the assays CHO-FSH-R cells were sub-cultured into 24-well plates until they reached confluency. Then, culture media was replaced with serum-free media, containing 250 µM 3-isobutyl-1-methyl-xanthine (IBMX) and divided in two groups. In the first group, increased amounts of Gonal-F[™], which is a recombinant source of human FSH, diluted in media collected from cultures of untransformed CHO cells (CHO CRL-9096) were added. In the second group, increased volumes of collection media from the recombinant colonies were added. Assays were conducted using four replicates of each sample. After 4 hours of incubation, cells were collected and lysed at room temperature for 10 min with 130 µl of extraction buffer (Appendix 1) and stored frozen at -20°C until assayed for luciferase activity. Luciferase activity was tested in 100 µl of lysed cells supernatant, which was added to 360 µl of luciferase assay mix (Appendix 1). Light emission in the reaction was read in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) after an automatic injection of 100 µl of luciferin solution (Appendix 1). Light emission during 20 seconds was integrated and a

maximum background of 500 relative light units based on a 0.5 second measuring time was subtracted. Response to Gonal-FTM by the CHO-FSH-R cells was used to derive the activity of the recombinant elk FSH.

4. CLONING AND EXPRESSION OF THE ELK PITUITARY GONADOTROPIN HORMONE GENES

4.1 Abstract

We report the nucleotide and deduced amino acid sequences of the elk (*Cervus elaphus*) pituitary glycoprotein hormone common α -subunit, and the FSH and LH β -subunits. These sequences were obtained by RT-PCR of pituitary gland polyadenylated RNA. A partial genomic fragment of the elk follicle stimulating hormone β -subunit was also amplified from genomic DNA and sequenced. These sequences demonstrated high sequence similarity to other related domestic species, including sika deer, ovine, bovine, porcine and equine. The presently reported cDNA sequences were used as probes for Northern blot analyses of pituitary gland and muscle samples from elk and sheep. Expression of mature mRNA transcripts of approximately 700, 1700 and 550 bases was observed in the pituitary gland but not in muscle tissue; for the glycoprotein hormone common α -subunit and FSH and LH β -subunits, respectively. These observations are consistent with reports for other related species. The genomic fragment of elk FSH β -subunit sequence revealed an intronic microsatellite of 6 CT repeats, which differs from the 19 CT repeats documented for the sheep genomic sequence.

4.2 Introduction

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are structurally related heterodimeric glycoproteins composed of two subunits, α and β ,

produced by the gonadotrope cells of the pituitary gland. They share a common α -subunit, while the β -subunit is unique for each hormone (Pierce and Parsons, 1981). FSH and LH are essential for ovarian follicular development, maturation of the oocyte, steroidogenesis and ovulation. In males, LH is involved in regulation of steroidogenesis in the Leydig cells, whereas FSH regulates spermatogenesis by binding to receptors in the Sertoli cells (Leung & Steele, 1992). The gene and protein structures of the pituitary glycoprotein hormones of a variety of vertebrates including, mammals, birds, amphibians and fish have been reported over recent years (Li and Ford, 1998). This information has been useful for the isolation and analysis of these genes in different species, identification and quantification of their mRNA and production of biologically active recombinant hormones. Analysis of the nucleotide and predicted amino acid sequences of the pituitary glycoproteins also provides further insight into the evolution of this family of hormones (Wallis, 2001). Little genetic information is available for cervid species. Recently, the gene structures of the pituitary glycoprotein common α -subunit (Li et al., 2001) and FSH β -subunit (Guan et al., 2002) were reported for the sika deer, a Japanese water deer. We are interested in studying seasonal expression of the gonadotropin genes in the elk, or wapiti (*Cervus elaphus*), a North American temperate seasonal breeder. We presently report the cloning and expression of the elk pituitary common glycoprotein α -subunit and the FSH and LH β -subunit cDNAs and their deduced amino acid sequences.

4.3 Results

Cloning of the pituitary glycoprotein common α -subunit cDNA

A 24 oligonucleotide forward primer was designed based on the reported ovine cDNA nucleotide sequence (Bello, et al., 1989), containing the putative common glycoprotein α -subunit start codon (Table 1). From the available gonadotropin sequences, ovine sequences were deemed to have the highest similarity to elk. Using the *Cervus elaphus* prion cDNA sequence, NCBI accession number AF016227 in a BLAST search the ovine sequence, NCBI accession number U67922 had a nucleotide similarity of 97% over 771 nucleotides. Thus, ovine gonadotropin sequences were used as the basis to design primers to amplify elk gonadotropin cDNAs. Using this primer and the 3' RACE reverse primer provided by the manufacturer, a DNA fragment of 691 base pairs (bp) was amplified (Figure 2). Three independent sequencing analyses of this fragment revealed high similarity, shown in Table 2, to the glycoprotein common α -subunit cDNA of sheep and other domestic species (Bello, et al., 1989; Goodwin et al., 1983; Kato et al., 1991; and Stewart et al., 1987). Comparison analysis suggests that 363 bp of this fragment represent the entire coding region, and 309 bp represent the 3' untranslated region (UTR) with the polyadenylated tail of the gene. A deduced 120 amino acid protein would be encoded by this sequence of nucleotides, shown in Figure 2.

Cloning of the pituitary FSH β -subunit cDNA

A 22 oligonucleotide forward primer, ending 5 bases upstream of the start codon, named forward FSH β -subunit (Table 1), was designed based on the reported bovine

FSH β -subunit cDNA (Maurer and Beck, 1986). A 21 oligonucleotide reverse primer, named reverse FSH β -subunit primer 1, was designed to amplify within the putative coding region of the reported ovine cDNA (Table 1). With these set of primers, a fragment of 398 bp was amplified, which demonstrated high sequence identity to FSH β -subunit cDNA of sheep and other domestic species (Mountford et al., 1989; Maurer and Beck, 1986; Kato 1988; and Saneyoshi et al., 1999). We considered this to be a truncated clone, since the sequence ended 36 bp before the putative stop codon of the sheep FSH β -subunit cDNA. Since we were having a difficult time amplifying the whole coding region of the FSH β -subunit sequence from pituitary gland RNA samples, we decided to try amplifying the last exons from genomic DNA. Therefore, to gain additional information on the last part of the elk FSH β -subunit gene coding region, a second set of primers were designed based on sheep genomic sequences (Guzman et al., 1991). A 23 oligonucleotide forward primer was designed based on intronic sequences located upstream of the second exon, and a 25 oligonucleotide reverse genomic primer was designed based on sequences located in the 3' UTR of the gene, named genomic forward FSH β -subunit and genomic reverse FSH β -subunit, respectively (Table 1). Using this second set of primers we amplified a DNA fragment of 397 bp that included genomic sequences, containing 6 intronic CT microsatellite repeats and the last 237 bp of the coding region. Based on this information, a 26 oligonucleotide reverse primer was designed, named FSH β -subunit reverse primer 2 (Table 1), which was used with the forward FSH β -subunit primer. A DNA fragment of 414 bp was amplified, shown in Figure 4, which demonstrated high similarity to the FSH β -subunit cDNA of sheep and other domestic species (Mountford et al., 1989; Guzman et al., 1991; Maurer and Beck,

1986; Kato 1988; and Saneyoshi et al., 1999). This fragment included 390 bp, representing the entire coding region, excluding the 5' and the 3' UTR of the FSH β -subunit gene. Four PCR fragments were sequenced, one truncated fragment from one animal, two genomic fragments from a second animal and one full-length fragment from a third animal. A deduced 129 amino acid protein would be encoded by this nucleotide sequence, shown in Figure 4.

Cloning of the pituitary LH β -subunit cDNA

A 23 oligonucleotide forward primer was designed based on the reported ovine cDNA, containing the putative LH β -subunit start codon (d'Angelo-Bernard et al., 1990). A 23 oligonucleotide reverse primer was designed to sequences located downstream of the putative stop codon, within the 3' UTR (Table 1). By using these primers a fragment of 524 bp was amplified, which revealed high similarity to the LH β -subunit cDNA of sheep and other domestic animals, (d'Angelo-Bernard et al., 1990; Virgin et al., 1985; Ezashi et al., 1990; Sherman et al., 1992). The elk LH β -subunit sequence was confirmed by sequence analysis of three fragments from three independent RT-PCR. This sequence includes the entire coding region, with an open reading frame of 366 bp, which would encode a deduced protein of 121 amino acids, shown in Figure 5.

Northern blot analysis

Expression of the elk gonadotropin genes in elk and sheep tissues is shown in the Northern blot autoradiograms of Figure 6. The common α -subunit, FSH β -subunit and

LH β -subunit genes are expressed in the elk pituitary gland as mature transcripts of approximately 700, 1700 and 550 bases, respectively. Transcripts of these genes migrated to the same position as the corresponding mRNA expressed in the sheep pituitary gland. Expression of the gonadotropin genes was not observed in elk muscle tissue.

| | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-----|---|-----|-----|-----|-----|-----|------|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| -19 | AGGCAGAGGACGAAGAGCC | | | | | | | | | | | | | | ATG | GAT | TAC | TGC | AGA | AAA | TAT | GCA | GCT | GTC | ATT | CTG | 36 |
| -24 | | | | | | | | | | | | | | | M | D | Y | C | R | K | Y | A | A | V | I | L | -13 |
| 37 | GCC | GTA | TTG | TCT | CTG | TTT | CTG | CAG | ATT | CTC | CAT | TCC | TTT | CCT | GAT | GGA | GAG | 87 | | | | | | | | | |
| -12 | A | V | L | S | L | F | L | Q | I | L | H | S | F | P | D | G | E | 5 | | | | | | | | | |
| | -1 / +1 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 88 | TTT | ATG | ATG | CAG | GGT | TGT | CCT | GAA | TGC | AAG | CTA | AAG | GAA | AAC | AAA | TAC | TTC | 138 | | | | | | | | | |
| 6 | F | M | M | Q | G | C | P | E | C | K | L | K | E | N | K | Y | F | 22 | | | | | | | | | |
| 139 | TCC | AAG | CCA | GAC | GCT | CCA | ATC | TAT | CAG | TGC | ATG | GGC | TGC | TGC | TTC | TCC | AGG | 189 | | | | | | | | | |
| 23 | S | K | P | D | A | P | I | Y | Q | C | M | G | C | C | F | S | R | 39 | | | | | | | | | |
| 190 | GCA | TAC | CCC | ACT | CCA | GCG | AGG | TCT | AAG | AAG | ACA | ATG | TTG | GTC | CCC | AAG | AAC | 240 | | | | | | | | | |
| 40 | A | Y | P | T | P | A | R | S | K | K | T | M | L | V | P | K | N | 56 | | | | | | | | | |
| 241 | ATC | ACC | TCG | GAA | GCC | ACA | TGC | TGT | GTG | GCC | AAA | GCG | TTT | ACC | AAG | GCC | ACA | 291 | | | | | | | | | |
| 57 | I | T | S | E | A | T | C | C | V | A | K | A | F | T | K | A | T | 73 | | | | | | | | | |
| 292 | GTG | ATG | GGA | AAC | GTC | AGA | GTG | GAG | AAC | CAC | ACC | GAC | TGC | CAC | TGC | AGT | ACT | 342 | | | | | | | | | |
| 74 | V | M | G | N | V | R | V | E | N | H | T | D | C | H | C | S | T | 90 | | | | | | | | | |
| 343 | TGT | TAT | TAT | CAC | AAA | TCT | TAG | AGGGTTTGCAATGGGCCGTGTTGGTGATGGCTGATTTGC | | | | | | | | | | 393 | | | | | | | | | |
| 91 | C | Y | Y | H | K | S | Stop | | | | | | | | | | | 96 | | | | | | | | | |
| 394 | CCAAAAGGAAAGCTAATTTGTCCAGTGTCTACGGCTTTGTGAGATAAAACCTCCTTCTCCTTGCCA | | | | | | | | | | | | | | | | | 460 | | | | | | | | | |
| 461 | TACCGCTTTTAACCTGCTTTGAGAATGTACTGCAGCTTCATTGCTTTTCTCCTTGCCCTACAATATA | | | | | | | | | | | | | | | | | 527 | | | | | | | | | |
| 528 | ATCAGCAGCCTCGATCTTTTCATTTGGAATGAAATATGGCATTTAGCATGACCATAAAAAGCTGGTT | | | | | | | | | | | | | | | | | 594 | | | | | | | | | |
| 595 | CCACTGGAAATAAAGTCTTTTAAATCATCAAAAAAAAAAACCTATAGTGAGTCGTATTAATTCGGA | | | | | | | | | | | | | | | | | 661 | | | | | | | | | |
| 662 | TCCGCGAAGGG | | | | | | | | | | | | | | | | | 672 | | | | | | | | | |

Figure 2. cDNA nucleotide and deduced amino acid sequences of the elk common glycoprotein α -subunit. The nucleotides of the 5' UTR are designated with negative numbers and the first nucleotide of the ATG translational start codon as +1. The forward custom primer was designed from nucleotide positions -19 to +5, overlapping the first two codons from the translational start site. The primers sequences are shown in bold. The polyadenylation signal is underlined. The end of the signal peptide and the beginning of the mature protein amino acid sequence is designated as -1/+1 boundary. The proposed leader sequence is 24 amino acids in length. The free α -subunit is proposed to be *O*-glycosylated at Thr⁴³, which is bolded and underlined. The α -subunit in the heterodimeric glycoprotein is putatively *N*-glycosylated at Asn⁵⁶ and Asn⁸², which are bolded and underlined. Proposed proper subunit folding involves 10 cysteines, shown in bold and located at positions 11, 14, 32, 35, 36, 63, 64, 86, 88 and 91 that form 5 disulfide bonds.

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2338 AGCAGTATTCAATCCCTGTCTCATTTTGATTACGCTACACAGAACTTCCAGACTACTGTAACATCAT 2405
2406 CTCTCTCTCTCTGTGTCTCCTAA ACC ACT CAG GAC TTG GTG TAC AGG GAC CCA GCG 2461
    39                               T  T  Q  D  L  V  Y  R  D  P  A    49
2462 AGG CCC AAC ATC CAG AAG ACA TGT GCC TTC AAG GAG CTG GTC TAC GAG ACG 2512
    50 R  P  N  I  Q  K  T  C  A  F  K  E  L  V  Y  E  T    66
2513 GTG AGA GTG CCC GGC TGC GCT CAC CGC GCA GAC TCC CTG CAC ACG TAC CCG 2563
    67 V  R  V  P  G  C  A  H  R  A  D  S  L  H  T  Y  P    83
2564 GTG GCC ACC GCG TGT CAC TGC GGC AAG TGT GAC AGC GGC AGC ACC GAC TGC 2614
    84 V  A  T  A  C  H  C  G  K  C  D  S  G  S  T  D  C   100
2615 ACC GTG CGG GGC CTG GGG CCC AGC TAC TGC TCC TTC AGC GAC GTC AGA GAG 2665
   101 T  V  R  G  L  G  P  S  Y  C  S  F  S  D  V  R  E   117
2666 TAA GGGACGGCGGCGCTCTGAGCTGCCCACCCTCACAGGCCAGGCCATCCAAGCTGTCTGTGTGTA 2713
    Stop
2732 CATG 2735

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Figure 3. Genomic nucleotide and deduced amino acid sequences of elk FSH β -subunit. The genomic nucleotide numbering is based on the NCBI S64745 ovine FSH- β genomic sequence. The forward custom primer was designed from nucleotide positions 2338 to 2361 and the reverse custom primer was designed from nucleotide positions 2710 to 2735. The primer sequences are shown in bold.

| | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-----|----------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
| -27 | AGCATCCACAGTTACCAAGTGCCAGG | | | | | | | | | | | | | | | ATG | AAG | TCC | ATC | CAG | TTC | TGC | TTC | CTT | TTC | 30 |
| -12 | | | | | | | | | | | | | | | | M | K | S | I | Q | F | C | F | L | F | -3 |
| 31 | TGT | TGC | TGG | AGA | GCA | ATC | TGC | TGC | AGA | AGC | TGC | GAG | CTG | ACC | AAC | ATC | ACC | 81 | | | | | | | | |
| -2 | C | C | W | R | A | I | C | C | R | S | C | E | L | T | <u>N</u> | I | T | 15 | | | | | | | | |
| | -1 /+1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 82 | ATC | ACG | GTG | GAG | AAA | GAG | GAA | TGC | AGC | TTC | TGC | ATA | AGC | ATC | AAC | ACC | ACG | 132 | | | | | | | | |
| 16 | I | T | V | E | K | E | E | C | S | F | C | I | S | I | <u>N</u> | T | T | 32 | | | | | | | | |
| 133 | TGG | TGT | GCG | GGC | TAC | TGC | TAC | ACC | CGG | GAC | TTG | GTG | TAC | AGG | GAC | CCA | GCG | 183 | | | | | | | | |
| 33 | W | C | A | G | Y | C | Y | T | R | D | L | V | Y | R | D | P | A | 49 | | | | | | | | |
| 184 | AGG | CCC | AAC | ATC | CAG | AAG | ACA | TGT | GCC | TTC | AAG | GAG | CTG | GTC | TAC | GAG | ACG | 234 | | | | | | | | |
| 50 | R | P | N | I | Q | K | T | C | A | F | K | E | L | V | Y | E | T | 66 | | | | | | | | |
| 235 | GTG | AGA | GTG | CCC | GGC | TGC | GCT | CAC | CGC | GCA | GAC | TCC | CTG | CAC | ACG | TAC | CCG | 285 | | | | | | | | |
| 67 | V | R | V | P | G | C | A | H | R | A | D | S | L | H | T | Y | P | 83 | | | | | | | | |
| 286 | GTG | GCC | ACC | GCG | TGT | CAC | TGC | GGC | AAG | TGT | GAC | AGC | GGC | AGC | ACC | GAC | TGC | 336 | | | | | | | | |
| 84 | V | A | T | A | C | H | C | G | K | C | D | S | G | S | T | D | C | 100 | | | | | | | | |
| 337 | ACC | GTG | CGG | GGC | CTG | GGG | CCC | AGC | TAC | TGC | TCC | TTC | AGC | GAC | GTC | AGA | GAG | 387 | | | | | | | | |
| 101 | T | V | R | G | L | G | P | S | Y | C | S | F | S | D | V | R | E | 117 | | | | | | | | |

Figure 4. cDNA nucleotide and deduced amino acid sequences of elk FSH β -subunit. The nucleotides of the 5' UTR are designated with negative numbers and the first nucleotide of the ATG translational start codon as +1. The forward custom primer was designed from nucleotide positions -27 to -6. The reverse custom primer was designed from nucleotide positions 362 to 387. Both primer sequences are shown in bold. The end of the signal peptide and the beginning of the mature protein amino acid sequence is designated at the -1/+1 boundary. The proposed leader sequence is 12 amino acids in length. The FSH β -subunit is proposed to be N-glycosylated at Asn¹³ and Asn³⁰, shown in bold and underlined. Proposed proper folding involves 12 half cysteines shown in bold and located at positions 9, 23, 26, 34, 38, 57, 72, 88, 90, 93, 100 and 110 that form 6 disulfide bonds.

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--------|---|-----|-----|-----|-----|-----|-----|-----|-----|------|-----------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|---|----|
| -17 | GGTACCAGGCACCAAGGATGGAG | | | | | | | | | | | | | | | | | ATG | CTC | CAG | GGG | CTG | CTG | CTG | TGG | CTG | CTG | CTG | 39 | | |
| -20 | | | | | | | | | | | | | | | | | | M | E | M | L | Q | G | L | L | L | W | L | L | L | -8 |
| 40 | GGC | GTG | GCC | GGG | GTG | TGG | GCT | TCC | AGG | GGG | CCA | CTG | CGG | CCG | CTG | TGC | CAG | 90 | | | | | | | | | | | | | |
| -7 | G | V | A | G | V | W | A | S | R | G | P | L | R | P | L | C | Q | 10 | | | | | | | | | | | | | |
| -1 /+1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 91 | CCC | ATC | AAC | GCC | ACC | CTG | GCG | GCT | GAG | AAG | GAG | GCC | TGC | CCC | GTC | TGC | ATC | 141 | | | | | | | | | | | | | |
| 11 | P | I | N | A | T | L | A | A | E | K | E | A | C | P | V | C | I | 27 | | | | | | | | | | | | | |
| 142 | ACT | TTC | ACC | ACC | AGC | ATC | TGC | GCC | GGC | TAC | TGC | CCC | AGC | ATG | AAG | CGG | GTG | 192 | | | | | | | | | | | | | |
| 28 | T | F | T | T | S | I | C | A | G | Y | C | P | S | M | K | R | V | 44 | | | | | | | | | | | | | |
| 193 | CTG | CCT | GCC | ATC | CTG | CCG | CCC | ATG | CCC | CAG | CGG | GTG | TGC | ACC | TAC | CAC | GAG | 243 | | | | | | | | | | | | | |
| 45 | L | P | A | I | L | P | P | M | P | Q | R | V | C | T | Y | H | E | 61 | | | | | | | | | | | | | |
| 243 | CTG | CGC | TTC | GCC | TCC | GTT | CGG | CTC | CCC | GGC | TGC | CCA | CCT | GGC | GTG | GAC | CCA | 294 | | | | | | | | | | | | | |
| 62 | L | R | F | A | S | V | R | L | P | G | C | P | P | G | V | D | P | 78 | | | | | | | | | | | | | |
| 295 | ATG | GTC | TCC | TTC | CCC | GTG | GCC | CTC | AGC | TGT | CAC | TGC | GGG | CCC | TGC | CGC | CTC | 345 | | | | | | | | | | | | | |
| 79 | M | V | S | F | P | V | A | L | S | C | H | C | G | P | C | R | L | 95 | | | | | | | | | | | | | |
| 346 | AGC | AGC | ACT | GAC | TGT | GGG | GGT | CCT | AGA | ACC | CAA | CCC | TTG | GCC | TGT | GAC | CAC | 396 | | | | | | | | | | | | | |
| 96 | S | S | T | D | C | G | G | P | R | T | Q | P | L | A | C | D | H | 112 | | | | | | | | | | | | | |
| 397 | CCC | CCG | CTC | CCA | GAC | ATC | CTC | TTC | CTC | TAA | GGATGCCCCACTTCAACCTCCCATGCC | | | | | | | 453 | | | | | | | | | | | | | |
| 113 | P | P | L | P | D | I | L | F | L | Stop | | | | | | | | 121 | | | | | | | | | | | | | |
| 454 | TCCTAACTCCCGGAGCCAGCAGACGCTCCTCCCTCCCTTCCCAATAAAGACTTCT | | | | | | | | | | | | | | | | | 509 | | | | | | | | | | | | | |

Figure 5. cDNA nucleotide and deduced amino acid sequences of elk LH β -subunit. The nucleotides of the 5' UTR are designated with negative numbers and the first nucleotide of the ATG translational start codon as +1. The forward custom primer was designed from nucleotide positions -17 to +6, overlapping the first two codons from the translational start site. The reverse custom primer was designed from nucleotide positions 485 to 509. Both primer sequences are shown in bold. The end of the signal peptide and the beginning of the mature protein amino acid sequence is designated as the -1/+1 boundary. The proposed leader sequence is 20 amino acids in length. The LH β -subunit is presumed to be *N*-glycosylated at Asn¹³, shown in bold and underlined. Proposed proper folding involves 12 cysteines shown in bold and located at positions, 9, 23, 26, 34, 38, 57, 72, 88, 90, 93, 100 and 110 that form 6 disulfide bonds.

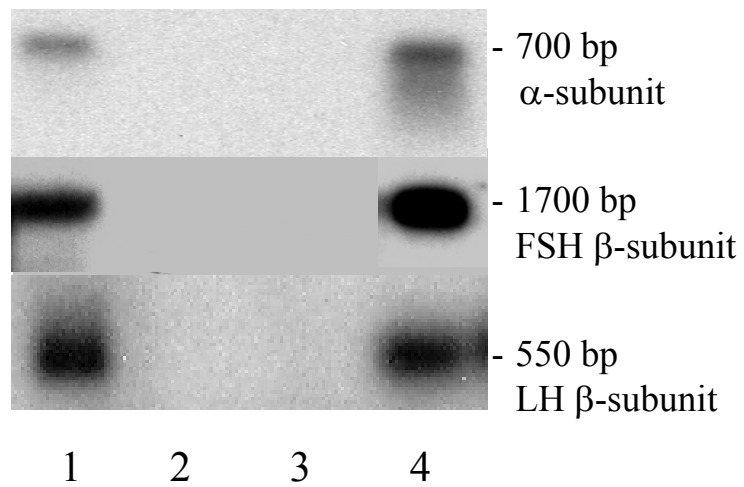


Figure 6. Northern blot autoradiographs of membranes hybridized with elk α -subunit, FSH and LH β -subunit cDNA probes. Lanes 1, 2, 3 and 4 were loaded with 10 μ g of total RNA from sheep pituitary gland, elk muscle, Chinese hamster ovary cells and elk pituitary gland, respectively.

| | Elk α -subunit 120 a.a. | Elk FSH β -subunit 129 a.a. | Elk LH β -subunit 141 a.a. |
|-----------|---------------------------------------|---|--|
| Sika deer | 99% (119/120) Li et al., 2001 | 96% (125/129) Guan et al., 2002 | — |
| Ovine | 95% (115/120) Bello et al., 1989 | 91% (118/129) Mountford et al., 1989 | 89% (126/141) d'Angelo-Bernard et al., 1990 |
| Bovine | 95% (116/120) Goodwin et al., 1983 | 90% (116/129) Maurer and Beck, 1991 | 88% (125/141) Virgin et al., 1985 |
| Porcine | 93% (112/120) Kato et al., 1991 | 89% (115/129) Li et al., 2000 | 85% (111/141) Ezashi et al., 1990 |
| Equine | 78% (94/120) Min et al., 1994 | 87% (113/129) Saneyoshi et al., 1999 | 78% (109/141) Sherman et al., 1992 |

Table 2. Pituitary gonadotropin deduced amino acid sequences similarities between elk and a sub-set of related species. Similarities are expressed as percent of identical amino acids over the total for the specie shown in brackets.

4.4 Discussion

We cloned and sequenced the elk pituitary gonadotropin α - and β -subunit cDNAs, and assessed expression of these genes by Northern blot analysis. The amplified elk α -subunit cDNA is 95% similar, but 62 bases shorter, than the sheep α -subunit cDNA reported by Bello et al. (1989). This discrepancy is due to the design of the forward α -subunit primer, which hybridized close to the start codon. Thus, our elk α -subunit cDNA clone has an incomplete 5' UTR, compared to the ovine α -subunit cDNA, but due to primer bias still includes the Kozak consensus sequence, (GCC) AorG CCATGG, required for translation in eukaryotes (Kozak, 1987) and the consensus polyadenylation signal, AATAAA, from nucleotide positions 603-608. During the course of this study, the glycoprotein α -subunit (Li et al., 2001) and FSH β -subunit (Guan et al., 2002) cDNA nucleotide sequences of the sika deer (*Cervus nippon*), a closely related species to the elk, were reported. The presently reported elk cDNA shares 99% nucleotide similarity to the glycoprotein α -subunit cDNA of the sika deer. Only one nucleotide difference was observed at position 11, where an adenine was reported for sika deer whereas in the elk we report a guanine. Using the cloned elk glycoprotein α -subunit cDNA as a probe, a mature transcript size of approximately 700 bases was identified, which is consistent with the sheep glycoprotein α -subunit mRNA length reported by Bello et al. (1989). Thus, we consider that this cDNA corresponds to the elk common pituitary glycoprotein α -subunit.

If the signal peptide of the elk α -subunit is of the same length as reported for ovine (Bello et al., 1989), the elk α -subunit cDNA would encode a mature protein of 96 amino acids. The nucleotide difference observed at position 11, translated into a

difference in the fourth deduced amino acid, we report a cysteine for the elk, whereas a tyrosine was reported for sika deer, ovine, bovine, porcine and equine (Bello et al., 1989; Goodwin et al., 1983; Kato et al., 1991; and Stewart et al., 1987). In addition, the forward primer we designed to amplify the elk α -subunit cDNA biased the first two deduced amino acids. Therefore, most likely the resulting deduced amino acids at positions 1 and 2 would be methionine and aspartic acid, which correspond to the first two amino acids reported for the other related species (Bello et al., 1989; Goodwin et al., 1983; Kato et al., 1991; and Stewart et al., 1987). The hydrophobic residues in the signal peptide, including valine, alanine, leucine, and isoleucine, remain the same in the elk as in sika deer, ovine, bovine and porcine. These are non-polar amino acids, which aid in the translocation of the nascent peptide to the rough endoplasmic reticulum. The amino acid difference observed is a conserved change since tyrosine and cysteine are both uncharged polar residues. Therefore, the difference observed may not affect the biological activity of the hormone, since the signal peptide is cleaved from the nascent peptide. The encoded elk α -subunit presently reported shares the highly conserved structural features found in the α -subunit of other mammalian species, including an *O*-glycosylation site at Thr⁴³, *N*-glycosylation sites at Asn⁵⁶ and Asn⁸², and 10 half cysteines, at positions 11, 14, 32, 35, 36, 63, 64, 86, 88, and 91, that form 5 intrachain disulphide bonds (Lapthorn et al., 1994).

The elk FSH β -subunit cDNA reported in this study is 98% similar, but 1175 bp shorter than the ovine FSH β -subunit cDNA reported by Mountford et al., (1989). This discrepancy is attributed to the forward FSH β -subunit primer, which hybridised at a site close to the start codon, and to the reverse FSH β -subunit primer 2, which hybridised to

the last 9 codons of the gene. Thus, this cDNA does not include 29 bp of the 5' UTR, nor the 1144 bp of the 3' UTR compared to the ovine FSH β -subunit cDNA, but includes 6 out of 10 of the nucleotides of the Kozak consensus sequence. The reported cDNA shares 98% nucleotide similarity to the FSH β -subunit cDNA of the sika deer. Using the cloned elk FSH β -subunit as a probe, a mature transcript size of approximately 1700 bases was identified, which is consistent with the reported size of the bovine FSH β -subunit mRNA (Kim et al., 1988). Therefore, we conclude that the reported cDNA corresponds to the elk FSH β -subunit.

The strategy used to clone the elk FSH β -subunit cDNA involved sequencing of a genomic fragment, from which we gained additional information on the structure of the gene. The elk FSH β -subunit genomic fragment presently reported contains 6 CT microsatellite repeats in the intronic sequence amplified. This differs from the genomic sheep FSH β -subunit sequence (Guzman et al., 1991), which contains 19 CT microsatellite repeats at the same location. If adequate variability in the number of these CT repeats were shown, this microsatellite could represent a marker for DNA fingerprinting in elk parentage testing (Talbot et al., 1996).

Five nucleotide differences within the coding region of the FSH β -subunit were observed between elk and sika deer. We presently report adenine, thymidine, guanine, cytosine and guanine, at the positions 10, 47, 208, 375 and 379, respectively; while guanine, cytosine, adenine, thymidine and adenine, were reported at the same positions in the sika deer sequence (Guan et al., 2002). These five nucleotide differences alter the sequence of four amino acids in the peptide chain. Thus, the elk FSH β -subunit cDNA would encode isoleucine, isoleucine, alanine and valine; while the reported sika deer

cDNA would encode for valine, threonine, threonine and isoleucine, at positions -9, 4, 58 and 115, respectively.

The nucleotide sequence of the FSH β -subunit cDNA was deduced from information obtained from three different animals, from which we amplified a truncated cDNA, a genomic fragment and a full-length cDNA. We observed discrepancies in the nucleotide sequences between these fragments. The truncated elk FSH β -subunit cDNA sequence differs from the full-length sequence at the nucleotide positions 79 and 218. We observed a cytosine and guanine in the truncated clone, whereas in the full-length cDNA we found adenine at both positions. These differences would mean changes in the encoded amino acids at residues 15 and 61. In the sika deer, a threonine was reported for residue 15, which is consistent with the truncated elk FSH β -subunit cDNA, but not with the full-length fragment, which would encode glutamic acid. In the sika deer, glutamic acid was reported for the residue 61, which is consistent with our observation in the full length and in the genomic fragments of the elk. The truncated elk FSH β -subunit cDNA exhibited glycine at the amino acid position 61. The genomic and full-length clones of the elk FSH β -subunit differ at amino acid residues 39 and 41. The genomic fragment would encode threonine and glutamic acid, while the full-length would encode tyrosine and arginine, at positions 39 and 41, respectively. In the sheep, tyrosine and arginine were reported at these positions, which is consistent with the elk FSH β -subunit full-length cDNA presently reported (Mountford et al., 1989). Therefore, the discrepancies reported could be due to amplification errors (Bracho et al., 1998) or to sequence polymorphisms between different animals. The full-length cDNA would encode a peptide chain of 129 amino acids, of which 117 correspond to the mature

protein. By aligning the conserved Asn¹³ and Asn³⁰, the length of the leader sequence of the elk FSH β -subunit would be 12 amino acids long, while in the sheep it was reported to have a leader sequence of 19 amino acids (Mountford et al., 1989). The deduced amino acid sequence presently reported retains the highly conserved mammalian FSH β -subunit structural features, including the *N*-glycosylation sites at Asn¹³ and Asn³⁰ and the 12 cysteines at positions 9, 23, 26, 34, 38, 57, 72, 88, 90, 93, 100 and 110 that form 6 intrachain disulphide bonds (Lapthorn et al., 1994).

The LH β -subunit cDNA we amplified is 96% similar, but 7 bp shorter than the ovine LH β -subunit cDNA reported by d'Angelo-Bernard et al. (1990). This discrepancy is due to the forward and reverse LH β -subunit primers, which did not target the complete 3' or 5' UTR sequences. This cDNA fragment includes 6 out of 10 of the nucleotides of the Kozak consensus sequence. Using the cloned elk LH β -subunit cDNA as a probe, a mature transcript size of approximately 550 bases was identified, which is consistent with the report for ovine (d'Angelo-Bernad et al., 1990). Thus, we consider that this cDNA corresponds to the elk LH β -subunit. This cDNA would encode a peptide chain of 121 amino acids, of which 101 correspond to the mature protein, assuming that the signal peptide of elk would be the same length as reported for ovine (d'Angelo-Bernard et al., 1990). The signal peptide is proposed to be the first 20 amino acids, which are cleaved to yield the mature protein. The forward LH β -subunit primer used compromises the first two translated amino acids, methionine and glutamic acid. These amino acids were reported in the same positions in ovine, bovine, porcine and equine LH β -subunit (d'Angelo-Bernard et al., 1990; Virgin et al., 1985; Ezashi et al., 1990; Sherman et al., 1992). Therefore, we speculate that despite the introduced bias of

the forward LH β -subunit primer, the elk LH β -subunit gene may encode for methionine and glutamic acid at positions one and two, respectively. The elk LH β -subunit presently reported shares the highly conserved structural features found among other mammalian species, including the *N*-glycosylation site at Asn¹³ and the 12 cysteines involved in proper intrachain disulphide bonds, at residues 9, 23, 26, 34, 38, 57, 72, 88, 90, 93, 100 and 110 (Lapthorn et al., 1994).

In summary, we cloned the cDNAs that correspond to the elk glycoprotein α -subunit and FSH and LH β -subunits. Sequence and Northern blot analyses demonstrate high sequence similarities between the gonadotropin hormones genes and their transcripts between elk, sika deer and sheep. These elk sequences represent a unique addition to the growing collection of the pituitary glycoprotein hormone sequences for various vertebrates, as cervid sequences are under represented in the NCBI GenBank database.

5. CLONING OF THE ELK TSH β -SUBUNIT cDNA AND SEASONAL EXPRESSION OF THE PITUITARY GLYCOPROTEIN HORMONE GENES

5.1 Abstract

We report the elk (*Cervus elaphus*) thyroid stimulating hormone (TSH) β -subunit cDNA cloning, nucleotide and deduced amino acid sequences. The TSH β -subunit cDNA was obtained by RT-PCR of polyadenylated pituitary RNA. The deduced elk TSH β -subunit peptide chain shares sequence similarities between 93 to 99% with the reported TSH β -subunit of ovine, bovine, porcine and equine. The TSH β -subunit gene is expressed in the elk pituitary gland as a mature transcript of approximately 600 bases, which corresponds to the size of the mRNA expressed in the sheep pituitary gland. Seasonal expression of the pituitary gonadotropin genes was investigated by Northern blot analyses. Samples of elk pituitary glands collected during the breeding season showed significant elevated steady state levels of common α -subunit and FSH and LH β -subunit gene expression, consistent with the seasonal reproductive cycling of this species. Samples collected later in the breeding season demonstrated decreased levels of the gonadotropin genes. TSH, measured during the transition into the breeding season, had similar levels of expression, regardless of the animal's reproductive status.

5.2 Introduction

Elk (*Cervus elaphus*) are members of the deer family and belong to an extraordinarily diverse group of ungulates, with over 25 recognized subspecies

distributed across Europe, Asia and North America. They range from the smaller Scottish red deer (*C. e. scoticus*) to the much larger North American elk, or wapiti, that can be further divided into three subspecies; *C. e. nelsoni*, *C. e. manitobensis* and *C. e. roosevelti* (Whitehead, 1993). Although sometimes considered as separate species, red deer and elk share the same karyotype ($2n = 68$) and are capable of producing fertile offspring (Gray, 1972).

Elk, as temperate cervids, are strict seasonal breeders. Most life-sustaining activities of cervids, such as feeding, locomotion, sleep and reproduction are photoperiod dependent. Therefore, activity of their reproductive organs exhibit annual changes, which are regulated by the length of daylight (Lincoln, 1971; Haigh et al., 1984). North American cervids start the mating season in autumn, in response to decreasing photoperiod (Adam et al., 1985). In the absence of pregnancy, deer hinds are polyestrous and are capable of exhibiting between four to nine continuous 17-19 day oestrous cycles over a 3-6 month period between autumn and spring (Guinness, 1971; Duckworth and Barrell, 1992; and Meikle and Fisher, 1996).

We are interested in studying expression of the pituitary glycoprotein genes, including the gonadotropins FSH and LH, and the structurally related TSH, as their regulation in the cervid have not been extensively studied at the molecular level. Each of these hormones is a heterodimeric protein consisting of a α -subunit, which is common to all, that is non-covalently bound to a hormone specific β -subunit (Pierce and Parsons, 1981). In the female, FSH and LH regulate ovarian development and steroidogenesis, and in the male, regulate spermatogenesis and steroidogenesis (Leung and Steele, 1992). Regulation of FSH and LH is tightly controlled by the hypothalamic

gonadotropin-releasing hormone (GnRH) and by the gonadal production of inhibin, activin and steroids. TSH, although not directly related to ovarian stimulation, controls thyroid gland function and regulation of metabolism and is autoregulated by a negative feedback mechanism (Abel et al., 2003).

It is plausible to hypothesize that cervids would exhibit seasonal expression of the genes encoding the reproductive hormones FSH and LH, while in contrast TSH could be expressed independently. Thyroid hormones permit the increase in response to estradiol negative feedback in ewes at the transition to anestrus (Thrun et al., 1997). In red deer, thyroxine treatment at the beginning of or during the non-breeding season was effective in suppressing plasma LH concentration, but this action of thyroid hormones did not occur during the breeding season (Anderson and Barrell, 1998). Thus, the seasonal regulation controlled by thyroid hormones is very critical during the transition from the breeding season into anestrus. We presently report the cloning of the elk pituitary TSH β -subunit cDNA and its deduced amino acid sequence. We also investigated seasonal expression of the pituitary glycoprotein common α -subunit and the FSH, LH and TSH β -subunit genes at the transition from anestrus into the breeding season.

5.3 Results

Cloning of the TSH β -subunit cDNA

A 26 oligonucleotide forward primer was designed to a site located 25 nucleotides upstream of the putative start codon of the gene, based on the reported ovine and bovine TSH β -subunit cDNA (Bockmann et al., 1997; Maurer et al., 1984). A 25 oligonucleotide reverse primer was designed to the putative last 5 codons of the ovine

TSH β -subunit cDNA (Table 1). By using these primers a 478 bp DNA fragment was amplified, shown in Figure 7, which revealed high similarity to the TSH β -subunit cDNA of sheep and other domestic animals (Bockmann et al., 1997; Maurer et al., 1984; Li et al., 1996; and Kania et al., 1996). This sequence, due to the bias of primer design including a stop codon, had an open reading frame of 417 bp, which would encode a deduced peptide chain of 138 amino acids, shown in Figure 7. Comparison analysis with a subset of other species suggests that the deduced protein chain of the elk β -subunit TSH is between 93 to 99% similar to a subset of other species (Table 3). The TSH β -subunit gene is expressed in the elk pituitary gland as a mature transcript of 600 bases, which corresponds to the size of the mRNA expressed in the sheep pituitary gland (Figure 8). Expression of the TSH β -subunit gene was not detected in samples of total RNA from elk muscle and brain, or Chinese hamster ovary cells, which were used as negative controls (Figure 8).

| | | | | | | | | | | | | | | | | | | | | | | | |
|-----|--|-----|-----|-----|-----------------|-----|-----|-----|-----|-----|-----|------------|------------|------------|------------|------------|------------|-----|-----|-----|-----|-----|-----|
| -52 | AGAGCTTTTAGTCTGGGTCATTACAACATCAGCTCACCAATGCAAAGTAAGCATG | | | | | | | | | | | | | | | | | | ACT | GCT | ATC | 12 | |
| -13 | | | | | | | | | | | | | | | | | | | M | T | A | I | -10 |
| 13 | TTC | CTG | ATG | TCC | ATG | ATT | TTT | GGC | CTT | GCA | TGT | GGA | CAA | GCA | ATG | TCT | TTT | 63 | | | | | |
| -9 | F | L | M | S | M | I | F | G | L | A | C | G | Q | A | M | S | F | 8 | | | | | |
| | -1 / +1 | | | | | | | | | | | | | | | | | | | | | | |
| 64 | TGT | ATT | CCA | ACT | GAG | TAT | ACT | ATG | CAT | GTC | GAA | AGG | AAA | GAA | TGT | GCT | TAC | 114 | | | | | |
| 9 | C | I | P | T | E | Y | T | M | H | V | E | R | K | E | C | A | Y | 25 | | | | | |
| 115 | TGC | CTA | ACC | ATC | AAC | ACC | ACC | ATC | TGT | GCT | GGA | TAT | TGT | ATG | ACA | CGG | GAT | 165 | | | | | |
| 26 | C | L | T | I | <u>N</u> | T | T | I | C | A | G | Y | C | M | T | R | D | 42 | | | | | |
| 166 | GTC | AAC | GGC | AAG | CTG | TTT | CTT | CCC | AAA | TAT | GCC | CTG | TCT | CAG | GAT | GTC | TGT | 216 | | | | | |
| 43 | V | N | G | K | L | F | L | P | K | Y | A | L | S | Q | D | V | C | 59 | | | | | |
| 217 | ACA | TAC | AGA | GAC | TTC | ATG | TAC | AAG | ACT | GCA | GAA | ATA | CCA | GGA | TGC | CCA | CGC | 267 | | | | | |
| 60 | T | Y | R | D | F | M | Y | K | T | A | E | I | P | G | C | P | R | 76 | | | | | |
| 268 | CAT | GTT | ACT | CCT | TAT | TTC | TCC | TAC | CCT | GTA | GCT | ATA | AGC | TGT | AAG | TGT | GGC | 318 | | | | | |
| 77 | H | V | T | P | Y | F | S | Y | P | V | A | I | S | C | K | C | G | 93 | | | | | |
| 319 | AAG | TGT | AAT | ACT | GAC | TAT | AGT | GAT | TGT | ATA | CAT | GAG | GCC | ATC | AAA | ACA | AAC | 369 | | | | | |
| 94 | K | C | N | T | D | Y | S | D | C | I | H | E | A | I | K | T | N | 110 | | | | | |
| 370 | TAC | TGT | ACC | AAA | CCT | CAG | AAG | TCC | TAT | GTG | GTG | GGA | TTT | TCT | ATC | TAA | TTT | 420 | | | | | |
| 111 | Y | C | T | K | P | Q | K | S | Y | V | V | G | F | S | I | Stop | | 125 | | | | | |
| 421 | TAATAG | | | | | | | | | | | | | | | | | | | | | 426 | |

Figure 7. cDNA nucleotide and deduced amino acid sequences of elk TSH β -subunit. The nucleotides of the 5' UTR are designated with negative numbers and the first nucleotide of the ATG translational start codon as +1. The forward custom primer was designed from nucleotide positions -52 to -27. The reverse custom primer was designed from nucleotide positions 401 to 426. Both primer sequences are shown in bold. The end of the signal peptide and the beginning of the mature protein amino acid sequence is designated as the -1/+1 boundary. The proposed leader sequence is 13 amino acids in length. The TSH β -subunit is presumed to be *N*-glycosylated at Asn³⁰, shown in bold and underlined.

| | | TSH β -subunit aa position | | | | | | | | | | | |
|---------------------------------|--|----------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| | | Number of aa (% of identity) | 5 | 15 | 33 | 43 | 69 | 76 | 88 | 109 | 120 | 121 | 122 |
| Elk | | 138 | A | T | I | V | A | R | I | T | V | V | G |
| Ovine Brockmann et al., 1997 | | 137 (99%) | A | M | I | V | A | R | I | T | V | V | G |
| Bovine Maurer et al., 1984 | | 134 (97%) | A | M | V | V | A | R | I | T | M | V | G |
| Porcine Li et al., 1996 | | 131 (94%) | A | M | I | F | V | H | I | T | V | L | E |
| Equine Kania et al., 1996 | | 129 (93%) | T | M | I | I | V | D | V | A | V | V | E |

Table 3. TSH β -subunit deduced amino acid sequence similarities between elk and a sub-set of related species. Similarities, shown in brackets, are expressed as percent of the identical amino acids over the total for the species. Amino acid positions where the elk sequence differs is shown. Non-identical amino acids are bolded.

Seasonal expression of the elk pituitary glycoprotein genes

The relative seasonal expression of the elk pituitary glycoprotein genes is shown in the Northern blot autoradiograms of Figure 8. The steady state levels of FSH β -subunit mRNA were 12- and 2-fold higher in the Oct 12th and Oct 28th samples, respectively, as compared to the Sept 1st sample. Steady state level of LH β -subunit mRNA was 2-fold higher in the Oct 12th sample as compared to the Sept 1st sample. In contrast, on Oct 28th the LH β -subunit mRNA was reduced to 1/3 the levels exhibited by the Sept 1st sample. Steady state levels of gonadotropin α -subunit mRNA were 3-fold higher in the Oct 12th sample as compared to the Sept 1st sample. In contrast, on Oct 28th the gonadotropin α -subunit mRNA was reduced to 1/2 the levels exhibited by the Sept 1st sample. The mRNA levels of pituitary glycoprotein hormones was similar between the anoestrous sheep pituitary gland collected Jan 19th and the elk pituitary gland collected Sept 1st. Irrespective of the date of sample collection, all animals had equal expression of TSH β -subunit mRNA.

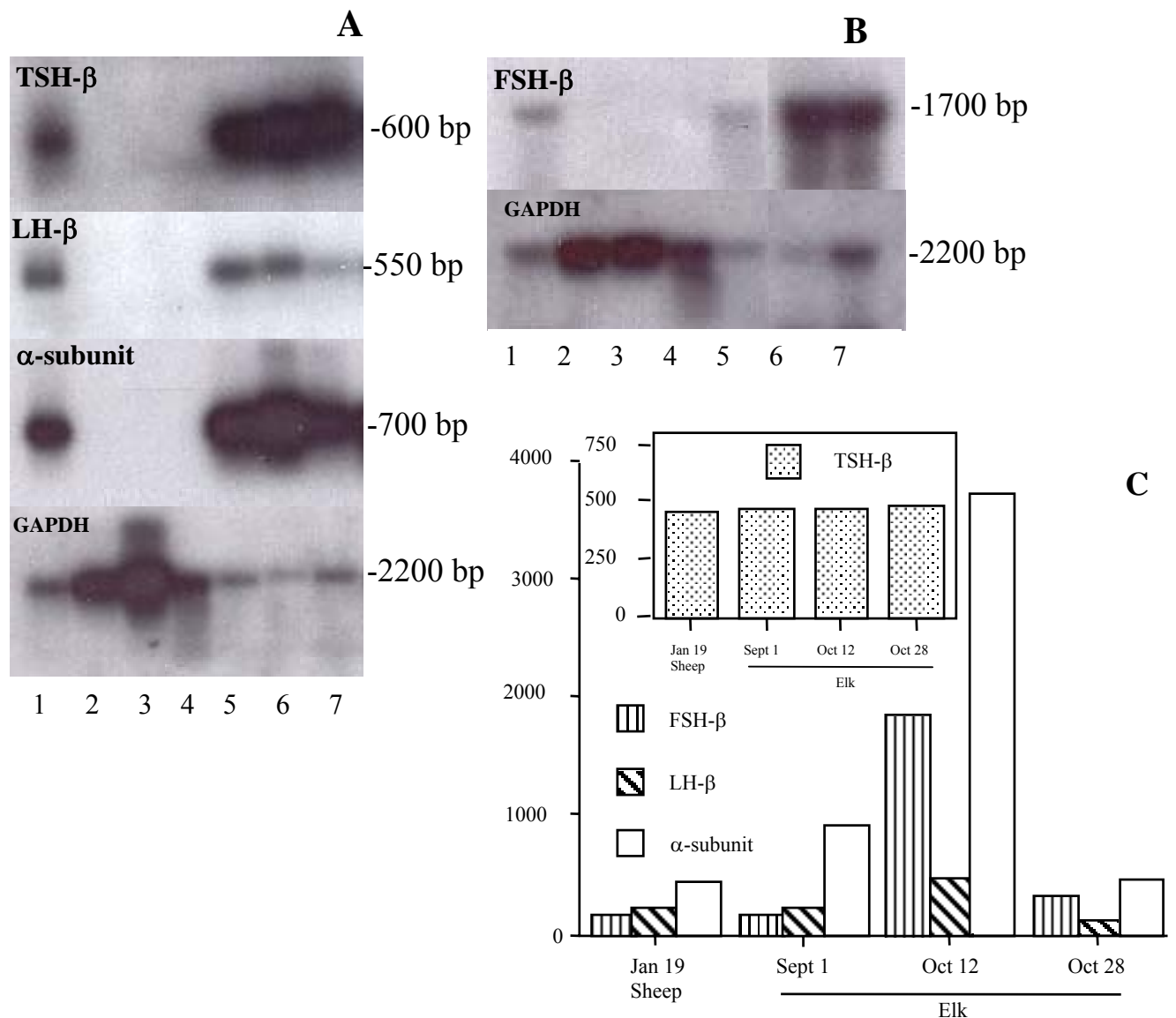


Figure 8. Autoradiograms of Northern blot analyses of sheep pituitary gland (1), elk muscle (2), Chinese hamster ovary cells (3), elk brain (4), and female elk pituitary glands collected Sept 1st, Oct 12th and Oct 28th, (5, 6, and 7), respectively. Membranes were probed with the cDNAs of TSH and LH β -subunits, the common α -subunit (Panel A) and the FSH β -subunit (Panel B). All membranes were reprobed with GAPDH cDNA. The numbers at the right of the figure indicate the approximate sizes in bases of the mature mRNA transcripts. Data were normalized for GAPDH mRNA levels in each sample and expressed relative to the control value (sheep). Panel C is a graphic representation of the values obtained, represented as arbitrary densitometric units.

5.4 Discussion

We cloned and sequenced the elk TSH β -subunit cDNA and investigated the seasonal expression of the common pituitary glycoprotein α -subunit, and the FSH, LH and TSH β -subunit genes in elk hinds. The amplified TSH β -subunit cDNA is 98% similar, but 70 bases shorter than the ovine TSH β -subunit cDNA reported by Brockmann et al. (1997). This discrepancy is due to the design of the forward primer, which did not target the complete 5' UTR sequence. Although this cDNA fragment does not have the entire 5' UTR, it does include 6 out of the 10 nucleotides of the Kozak consensus sequence needed for translation (Kozak, 1987). Thus, we consider that this fragment corresponds to the elk TSH β -subunit cDNA. This cDNA would encode a peptide chain of 138 amino acids, of which 125 correspond to the mature protein. The leader sequence for the elk TSH β -subunit is proposed to be 13 amino acids in length, determined by aligning with the conserved Asn³⁰ residue of the related species. The length of the elk signal peptide is shorter than the porcine signal peptide, which was reported to be 20 amino acids long (Li et al., 1996). For the 15th amino acid we report a tyrosine for elk, whereas methionine was reported for sheep (Brockmann et al., 1997). This is not a conserved change, as tyrosine is an aromatic amino acid while methionine is a sulfur amino acid, which occurs in the mature protein. Moreover, the reverse primer used compromises the last 5 deduced amino acids and the putative stop codon. Therefore, we cannot speculate on the nature of these last five amino acids in the elk TSH β -subunit. The differences in amino acid sequence detailed in Table 3 may result in differences in the functionality of the hormone between the species.

By using the cDNAs of the α -subunit, the FSH and LH β -subunits (Clark et al 2004a, submitted), and the presently reported TSH β -subunit, as probes, we investigated the seasonal expression of the pituitary glycoproteins in the female elk (Clark et al., 2004b, submitted). The relative changes in the β -subunit mRNA levels observed were consistent with the presumed reproductive status of the animals investigated. The Sept 1st sample showed low levels of FSH and LH β -subunit mRNA, which was similar to the levels of expression of the same genes in the anoestrous sheep (Huchkowsky et al., 2002). This observation is consistent with the information indicating that the sample corresponds to an animal that did not show evidences of estrous, and was taken before the beginning of the elk reproductive season.

The Oct 12th sample shows elevated levels of FSH and LH β -subunits, and was taken from a cycling animal during the middle of the reproductive season for the species. Elevated levels of FSH and LH β -subunits mRNAs were observed in this animal, which is coincident with the increased transcriptional activity of the pituitary gonadotropes during the reproductive season (Landefeld & Kepa, 1984; Barrell et al., 1992). These observations are consistent with the reported changes in the gonadotropic cells in the pituitary gland of the wild Sika deer and other temperate cervids. Changes in the activity of the pituitary gonadotropes were reported to be correlated with the high ovarian activity observed during the mating period of the Sika deer (Yamaji et al., 1994; and Yamauchi et al., 1984).

The sample from Oct. 28th was taken from an animal that was likely pregnant, as it was in the same pen with a bull. With adequate nutrition and when other environmental and social factors are not limiting, elk exhibit high fertility, with >95% of

mature females bearing singleton calves each year (Clutton-Brock, 1982). Thus, it is reasonable to speculate that the elevated levels of steroids typical during pregnancy resulted in a lower expression of the gonadotropin genes (Barrell et al., 1992).

Expression of the FSH and LH β -subunit genes is also consistent with the observed elevated levels common glycoprotein α -subunit mRNA. The highest steady state level of glycoprotein α -subunit mRNA was observed in an animal during the breeding season, which also demonstrated the highest levels of FSH and LH β -subunits mRNA. Expression of the glycoprotein α -subunit gene is needed not only for the synthesis of FSH and LH, but also for synthesis of TSH. Therefore, it is reasonable to speculate that the elevated levels of the common α -subunit observed during the reproductive season respond to the requirements for the synthesis of all the glycoprotein hormones produced in the pituitary gland. Moreover, it is known that an excess of α -subunit mRNA is normally produced in pituitary gland (Counis et al, 1982). Overall, the results of the expression of the gonadotropin genes are consistent with the cycling nature of the elk reproductive season.

Thyroid stimulating hormone β -subunit steady state mRNA levels were shown to have similar expression in all the samples studied. Since TSH is not directly involved in regulating entry into the breeding season; it was therefore speculated that TSH β -subunit expression would not be altered by the reproductive status of the animals under study. When the expression of TSH β -subunit mRNA was investigated among ovariectomized female rats receiving estrogen treatments, little to no change was detected (Maurer 1984).

In summary, the cloned elk TSH β -subunit cDNA presently reported shares high similarity with the bovine and sheep published reports. This information represents an important addition to the collection of sequences for the cervids in the gene database (Clark et al., 2003). The transcript levels of TSH were similar regardless of the reproductive status of the animal. The present study proposes there is transcriptional quiescence of the pituitary gonadotropes during anoestrous and pregnancy, while there is a dramatic increase in expression of the gonadotropic genes during the breeding season in the elk.

6. EXPRESSION OF THE ELK FSH GENES IN CHINESE HAMSTER OVARY (CHO) CELLS

6.1 Abstract

We sub-cloned the previously described elk common gonadotropin α -subunit and elk FSH β -subunit cDNAs into the double site mammalian expression vector, pBudCE4.1. A second construct was engineered in which the endogenous stop codon of the elk FSH β -subunit cDNA was deleted and replaced by nucleotides encoding a thrombin recognition site and an in-frame *XhoI* site, which allows incorporation of additional amino acids, including the V5 epitope and 6 consecutive histidine residues. This strategy facilitates purification of the recombinant protein by either immunoaffinity or nickel column chromatography. Separate CHO transfections were conducted with these two different constructs, from which two groups of cell lines were obtained; the first, expressing the wild-type elk FSH cDNA sequences and the second, expressing the wild-type elk common gonadotropin α -subunit and engineered elk FSH β -subunit cDNA constructs. Samples of total RNA from these cell lines were analysed by Northern blot for α - and FSH β -subunit gene expression. The colony with the highest expression of FSH genes from each group was selected for further culture. Culture media from each of these colonies was tested for FSH bioactivity in an *in vitro* assay based on CHO cells expressing the human FSH receptor and luciferase reporter gene. Culture media from either cell line induced a 2-fold increase in FSH receptor activity, as compared to media collected from untransfected CHO cells. In a parallel assay, the bioactivity of

recombinant human FSH (Gonal-FTM) was tested and used as a standard to derive the activity of the recombinant elk FSH. Based on this assay we found that both cell lines produced an equal amount of elk FSH, estimated to be equivalent to ~100 mIU/ml of recombinant human FSH. In conclusion, the elk FSH α - and β -subunit cDNAs were cloned and expressed in CHO cells. The strategies used translated into two biologically active hormones as ascertained by an *in vitro* bioassay. The addition of a short stretch of extra amino acids to the C-terminus of the native FSH β -subunit appear not to affect bioactivity of the recombinant elk FSH.

6.2 Introduction

Follicle stimulating hormone is a heterodimeric glycoprotein produced by the gonadotrope cells of the anterior pituitary gland of vertebrates, composed of an α - and β -subunit. The α -subunit, is common to other glycoproteins of the same family, including luteinizing hormone (LH), thyroid stimulating hormone (TSH), also produced in the pituitary gland, and the chorionic gonadotropins (CG) produced by the placenta of primates and equids. The common α -subunit is non-covalently associated with a hormone specific β -subunit to form an active molecule (Pierce and Parsons, 1981). Both subunits are encoded by separate genes, which have been cloned from a variety of species, including elk (Clark et al., 2004a, submitted), ovine (Bello et al., 1989; Mountford et al., 1989), bovine (Goodwin et al., 1983; Maurer and Beck, 1986), rat (Godine et al., 1982; Maurer et al., 1987), and human (Fiddes and Goodman, 1979; Jameson et al., 1988). Expression of these genes in Chinese hamster ovary (CHO) cells resulted in cell lines producing bioactive recombinant FSH of ovine (Mountford et al., 1994), rat (Hakola et al., 1997) and human (Keene et al., 1989; Chotigeat et al., 1994;

Gebert and Gray, 1994) species. These recombinant FSH molecules have been shown to have very similar oligosaccharide composition, compared to those of pituitary origin (Szkudlinski et al., 1993; Hakola et al., 1997). Recombinant FSH has been successfully used in superovulatory programs, and has provided a valuable tool for studying structure and function of glycoprotein hormones.

This is a report on the cloning and expression of FSH genes from the North American elk or wapiti. Elk is a farmed species in Canada, and is a close relative of the red deer and sika deer, farmed in New Zealand and Japan respectively. Along with many other closely related deer species, which are kept in zoos due to decreased numbers in the wild. The objective of this investigation is to produce recombinant elk FSH. It is believed that availability of this hormone would be advantageous for superovulation and application of advanced reproductive technologies in elk, to be used to rapidly increase the numbers of healthy and genetically superior individuals.

6.3 Results

Northern Blot Analyses of Transfected CHO colonies

The results of the Northern blot analyses of 6 colonies from each group of transfected CHO cells are shown in Figure 9. One colony from each group of cells expressed high levels of elk gonadotropin α -subunit and FSH β -subunit transcripts. The elk gonadotropin α -subunit and FSH β -subunit mRNAs migrated at positions corresponding to approximately 800 and 970 bases, respectively. Unequal levels of expression were observed between subunits (Figure 9). Expression of either subunit was not observed in untransfected CHO cells (Figure 9).

In Vitro FSH Bioassay

The results of the FSH bioassays of one colony from each group of transfected CHO cells are shown in Figure 10. Collection media from transformed cells increased luciferase activity in dose-dependent manner. A 2-fold maximum increase of luciferase activity, as compared with untransfected cells, was observed for both, the pBudCE4.1-elk- α - β -subunit and pBudCE4.1-elk- α - β -His transformed colonies. This activity was equivalent to ~100 mIU/ml, as compared to Gonal-FTM in the same assay (Figure 11).

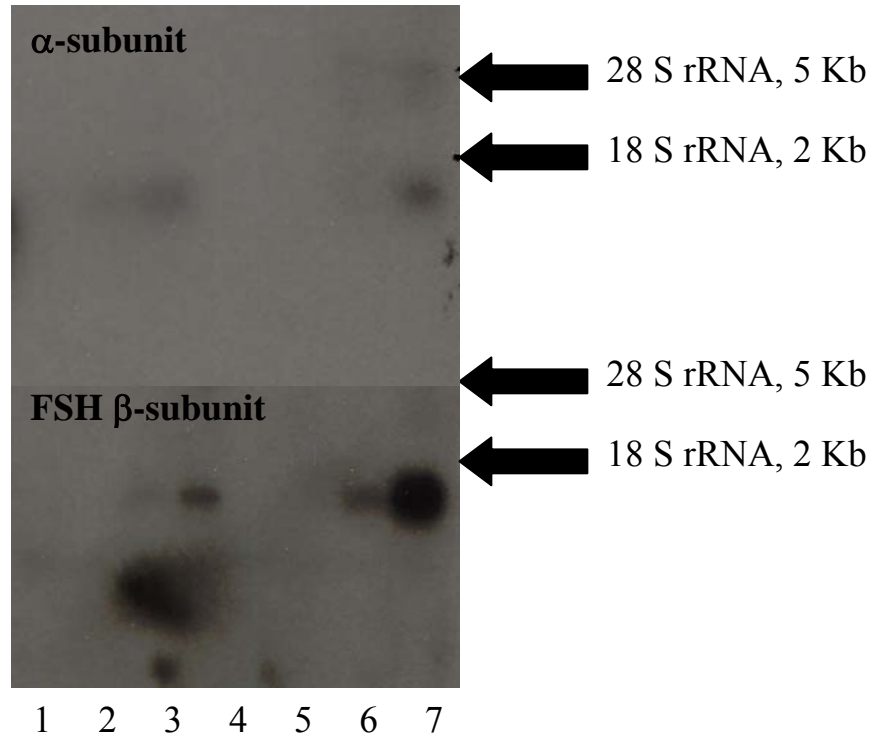


Figure 9A. Northern blot analysis of 6 colonies transfected with the native elk FSH genes. Lanes 1, 2, 3, 5, 6, and 7 represent 10 μ g of total RNA from 6 CHO colonies transfected with pBudCE4.1-elk- α - β -subunit vector. Lane 4 represents 10 μ g of total RNA from untransfected CHO CRL-9096 cells. The top and bottom panels represent autoradiographs hybridized with the elk gonadotropin α -subunit and FSH β -subunit cDNAs, respectively. The colony in lane 7 was cultured for further analysis.

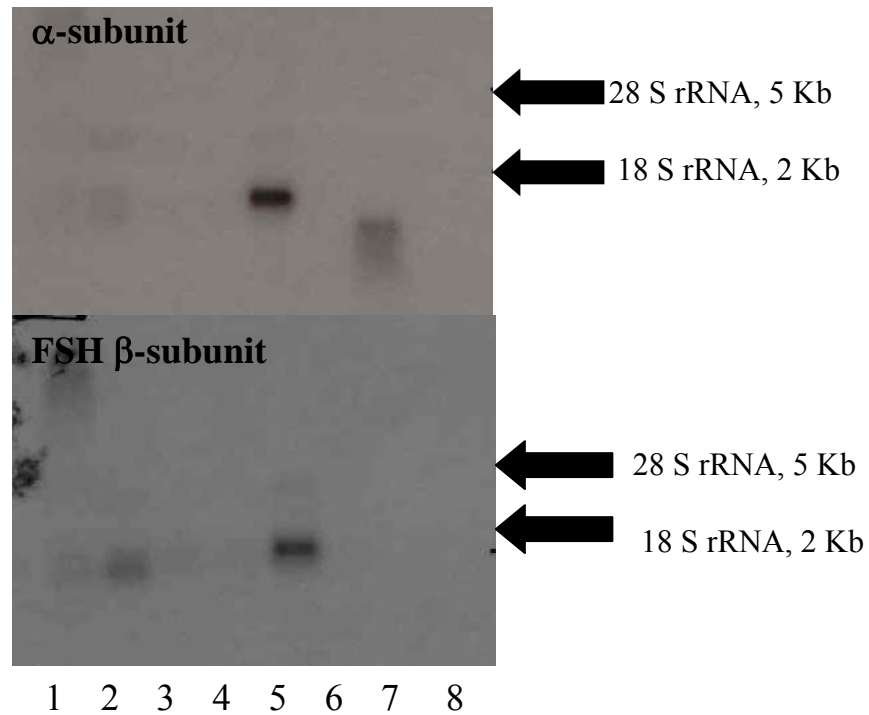


Figure 9B. Northern blot analysis of 6 CHO colonies transfected with the native elk gonadotropin α -subunit and an engineered FSH β -subunit genes. Lanes 1, 2, 3, 4, 5, 6 and 7 represent 10 μ g of total RNA from 6 CHO colonies transfected with pBudCE4.1-elk- α - β -His vector. Lane 8 represents 10 μ g of total RNA from untransfected CHO CRL-9096 cells. The top and bottom panels represent autoradiographs hybridized with the elk gonadotropin α -subunit and FSH β -subunit cDNAs, respectively. The colony in lane 4 was cultured for further analysis.

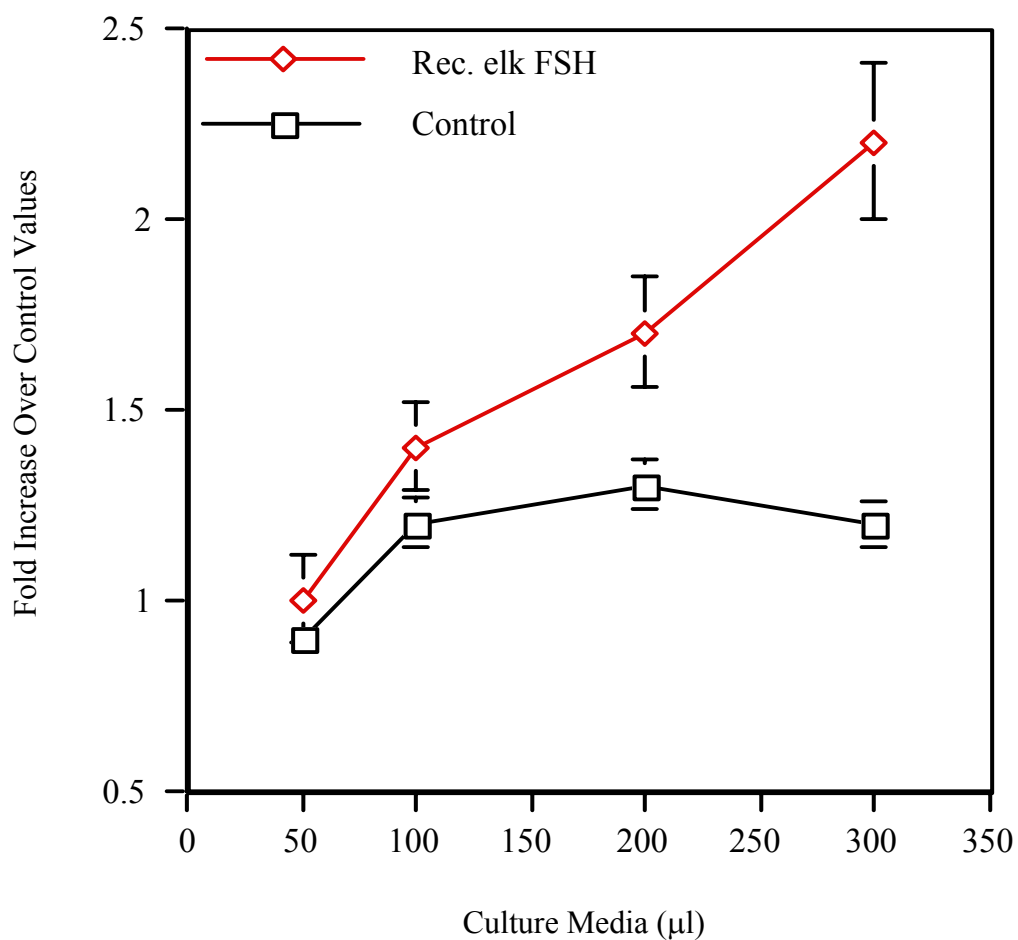


Figure 10. Bioactivity of recombinant elk FSH. Increasing volumes of culture media from CHO cells expressing the elk FSH genes or untransfected CHO CRL 9096 cells was exposed for 4 hours to CHO cells expressing the human FSH receptor and luciferase genes (CHO-hFSHR-Luc cells). Luciferase activity was assayed as described in materials and methods. The data represents the fold increase over mean control values of CHO-hFSHR-Luc cells treated with only treatment media and standard error for four replicates.

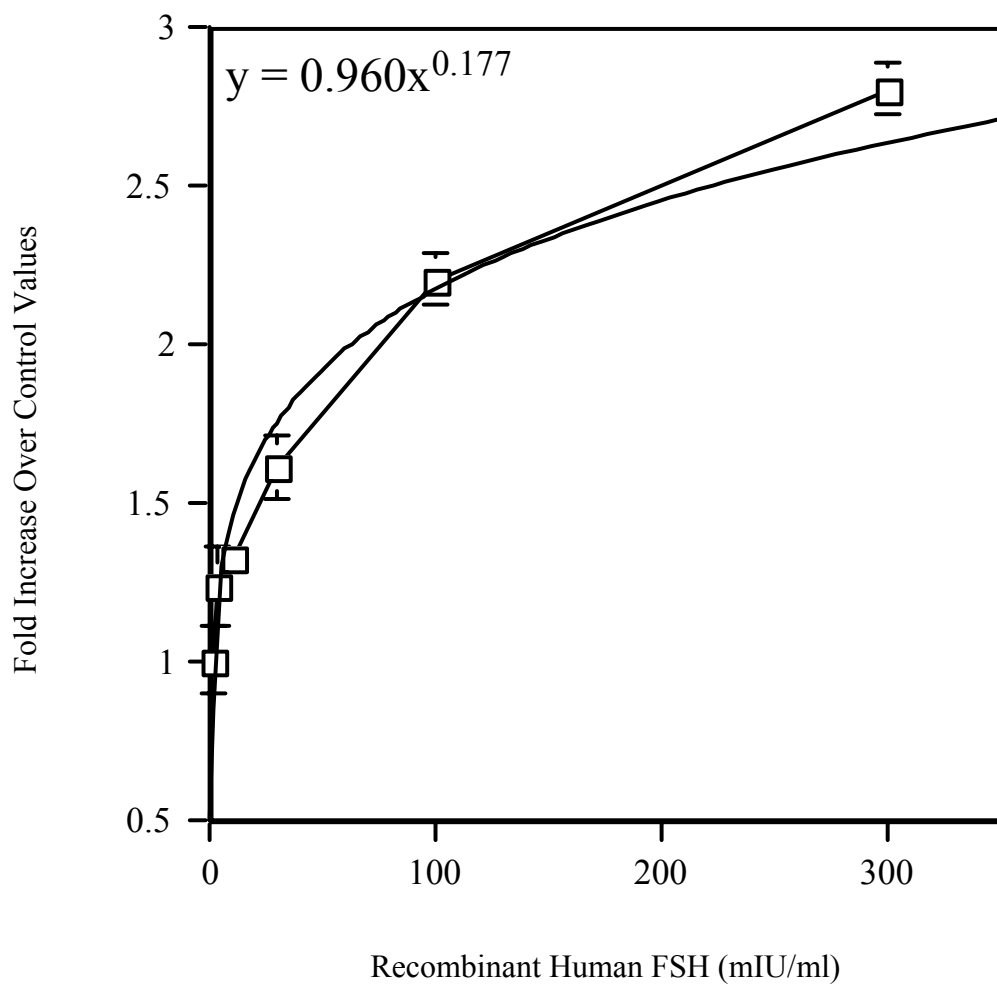


Figure 11. Effect of recombinant human FSH (Gonal-FTM) on CHO cells expressing the human FSH receptor and luciferase genes (CHO-FSH-R). CHO-FSH-R cells were exposed to increasing quantities of Gonal-FTM standard. After 4 hours of incubation, luciferase activity was assayed as described in materials and methods. Data represents the fold increase over control values and standard error for four replicates. The curve fit line was used to generate the equation to calculate the equivalent mIU of recombinant FSH in our cell culture samples.

6.4 Discussion

This is a report on the production of recombinant elk FSH using CHO cells expressing the FSH genes. In previous studies conducted in our laboratory, expression of the gonadotropin subunit genes was attempted, transfecting cells with two different single expression vectors. This procedure required extensive screening of a large number of clones in order to identify cells expressing both subunits. In the present study, CHO cells were transfected with a dual expression vector, pBudCE4.1. This strategy was aimed at obtaining simultaneous genomic integration, balanced expression of both subunits and enrichment in the number of positive clones. Although a large number of colonies with both subunits were obtained, unbalanced expression was still observed (Figures 9A and 9B). The α - and β -subunits were cloned downstream of the CMV and hEF-1 α promoters, respectively, which are not equally active (Invitrogen, Technical Service, personal communication). Thus, it is reasonable to think that genes which were under the control of different promoters resulted in unbalanced expression. An alternative interpretation is that the gonadotropin genes may not necessarily need to be expressed in a balanced manner, since normally in the pituitary gland; the α -subunit is expressed in excess, while FSH β -subunit expression limits hormone production (Greenberg et al., 1991). These observations raised speculations on the potential intrapituitary role of free α -subunit, and has been suggested that it may regulate synthesis/secretion of other hormones, including PRL (Counis et al., 1982).

It is known that the relatively long 3' UTR of the FSH β -subunit transcript contains several destabilizing sequence motifs, which accounts for the rapid degradation of its mRNA (Mountford et al., 1992; Brown et al., 2001). Therefore, in an effort to

optimize message translation, the elk gonadotropin α -subunit cDNA was cloned with its 3' UTR containing its endogenous stop codon and polyadenylation signal; but the FSH β -subunit cDNA was cloned without its 3' UTR. The strategy used was also intended to facilitate subsequent purification of the recombinant hormone. It is known that the common α -subunit can be secreted as a single subunit, whereas the β -subunit is only found in its heterodimeric form (Holdstock and Burrin, 1994). Thus in order to facilitate purification of heterodimeric recombinant protein from culture media, the FSH β -subunit, rather than the α -subunit, was targeted for the addition of a C-terminal extension. This extension contained an enzymatic target for thrombin, followed by 6 consecutive histidine residues. Thus, in the eventuality that the additional amino acid added to the β -subunit affect biological activity, the thrombin enzyme could be used to digest the C-terminal extension. This approach has been used successfully applied for the production and purification other recombinant proteins (van Bemmelen et al., 2000). This strategy is based on the nickel binding property of the histidine tag. Thus, by using an affinity column chromatography with a nickel-bead based matrix, dimeric recombinant protein with the histidine tag can be purified.

For targeting with the histidine tag, we chose the β -subunit, because modification of the α -subunit C-terminus impairs signal transduction (Marichatou et al., 2000); and the C-terminus was used because β -subunit N-terminus deletions strongly inhibit its ability to combine with the α -subunit (Huang et al., 1993). In addition, engineered FSH molecules in which the α - and β -subunits are expressed as a single molecule are capable of stimulating receptor signal transduction (Sugahara et al., 1995; Sugahara et al., 1996; Grossman et al., 1997). Both recombinant product obtained in this investigation resulted

equally active, supporting the concept that the C-terminus of the β -subunit may not be involved in dimer formation or signal transduction.

CHO cells have been used to express the glycoprotein genes of other mammalian species, which resulted in biologically active hormones (Olijve et al., 1996; Gerbert and Gray 1994; Peroni et al., 2002; Mountford et al., 1994; Hakola et al., 1997). Thus it appears to be the most suitable host expression system available to produce FSH. Expression of the gonadotropin genes in this system resulted in glycoproteins with adequate post-translational modifications required for optimal biological activity of the hormone. These include processing, proper disulfide bonds for peptide folding and an eukaryotic pattern of glycosylation. Recombinant pituitary glycoproteins have also been produced in non-mammalian systems, such as yeast, plant and insect cells (Richard et al., 1998; Dirnberger et al., 2001; Kato et al., 1998). Although these systems are more efficient than CHO cells, they are not well suited for the production of glycoproteins, since they exhibit non-mammalian pattern of glycosylation, which resulted in hormones with reduced biological activity.

Serum-free culture media was collected from each different recombinant elk FSH colony for biological analysis using a CHO stable cell line expressing the human FSH receptor and the gonadotropin α -subunit promoter driving the expression of the luciferase gene (Albanese et al., 1994). The collection media from the cells expressing the elk FSH constructs was able to increase luciferase activity by 2-fold, compared to collection media from untransfected CHO cells. The bioassay used to assess activity of recombinant elk FSH is based on a cell line expressing the human FSH receptor. Therefore, it is reasonable to speculate that since this a heterologous system, activity of

the elk recombinant may be underestimated. This speculation is based on information emerging from studies using recombinant human FSH in heifers (Takagi et al., 2001). Hormone specie differences, such as binding affinity for the receptor, activation of the bovine receptor, or differences in the degradation time in the cows were considered to be the causes for the sub-optimal superovulatory responses observed in these studies.

In conclusion, stably transfected CHO cells expressing the elk FSH genes from a dual expression vector are capable of producing a biologically active hormone as ascertained by an *in vitro* cell based assay. Addition of a short stretch of extra amino acids to the C-terminus of the FSH β -subunit does not negatively affect FSH subunit association, FSH receptor binding or signal transduction

7. SUMMARY OF RESULTS AND GENERAL CONCLUSIONS

- The common glycoprotein α -subunit and the specific FSH, LH and TSH β -subunit cDNAs from elk were cloned by RT-PCR. Sequence analysis of these cDNAs revealed that the elk pituitary glycoproteins share high sequence similarities with other related domestic species, such as sika deer, ovine, bovine, porcine and equine. Differences were observed within the FSH- β sequence, which may represent an allelic version of the same gene, which could be used as a marker in parentage mapping. This information is an addition to the growing collection of the pituitary hormones genes for various vertebrates. It also represents an advance that will facilitate evolutionary and phylogenic studies of the gonadotropin genes.
- Northern blot analysis of pituitary samples, using these cDNAs as probes, suggest that the common α -subunit gene is expressed at the highest steady state levels compared to the other genes investigated. Regardless of season the common α -subunit gene was expressed, but showed an increase during the breeding season. The FSH and LH β -subunit genes were expressed at low levels during presumed anestrus and pregnancy, but steady state levels significantly increased during estrus of the female elk. In contrast, the observed steady state levels of TSH β -subunit gene expression were similar regardless of the animal's reproductive status for the single sample animals we investigated;

- The elk common α -subunit and the native, or an engineered, FSH β -subunit cDNAs were cloned into the mammalian expression vector pBudCE4.1. CHO cells transfected with these vectors expressed the FSH subunit transcripts and produced recombinant products with FSH activity *in vitro*. Cells expressing the native construct and the engineered version of the FSH β -subunit showed similar *in vitro* biological activity. The engineered version of the FSH β -subunit expressing additional amino acids, including a 6X histidine tag attached to the C-terminus of the molecule, will facilitate purification of pure recombinant elk FSH from the cell culture media;
- Overall, this work represents an advance in the understanding of the seasonal regulation of the elk pituitary glycoproteins at the molecular level. This information and the availability of recombinant FSH will be required for the application of advanced reproductive technologies required for the rapid expansion of healthy and genetically superior animals, which represent an economic value for the elk industry.

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APPENDICES

Appendix 1

BACTERIAL CULTURE MEDIA

Luria Bertani (LB) media for bacterial culture

10 g bacto-tryptone

5 g bacto-yeast extract

10 g NaCl

pH 7, NaOH

Autoclave 20 minutes at 15 lbs/sq.in.

Ampicilin 50 µg/ml or if Zeocin 50 µg/ml, and lower salt content to 5 g/L of NaCl.

SOLUTIONS FOR WHOLE BLOOD DNA EXTRACTION

Triton/sucrose lysis buffer

50 mM MgCl₂

10 mM Tris-Cl, pH 7.5

1% Triton X-100

0.32 M sucrose

Salting out/lysis buffer

10 mM Tris-Cl, pH 7.5

0.4 M NaCl

2 mM disodium ethylenediamine-tetra acetate (EDTA), pH 8

SOLUTIONS FOR SELECTION OF POLYADENYLATED RNA

Autoclave below solutions, then add 0.1% sodium dodecyl sulphate (SDS)

Loading buffer A

40 mM Tris, pH 7.4

1 M NaCl

1 mM EDTA

Loading buffer B

20 mM Tris, pH 7.4

0.1 M NaCl

1 mM EDTA

Elution buffer

10 mM Tris, pH 7.4

1 mM EDTA

SOLUTIONS FOR LUCIFERASE ASSAY

Extraction buffer

1% Triton X-100 in water

Just before assay add, 1 mM dithiothreitol (DTT)

Luciferase assay mix

25 mM Gly-gly, pH 7.8 [with 5M K(OH)]

15 mM MgSO₄

4 mM EGTA

15 mM PO₄HK₂, pH 7.8

Just before assay add, 2 mM adenosine triphosphate (ATP) and 1 mM DTT

Luciferin solution

Assemble just before assay

0.2 mM Luciferin (keep individual stocks frozen at -80°C in gly-gly)

0.4 M Gly-gly, pH 7.8 [with 5M K(OH)]

1 mM DTT

SOLUTIONS FOR NORTHERN HYBRIDIZATION

Hybridization solution

7 % SDS

1 mM EDTA, pH 8

0.263 M Na₂HPO₄

1 % bovine serum albumin (BSA)

RNA loading buffer

0.75 ml formamide

0.15 ml 10x 3-(N-Morpholino) propanesulfonic acid (MOPS)

0.24 ml formaldehyde

0.1 ml glycerol

0.3 mg/ml bromophenol blue

0.18 ml water

10X MOPS buffer

0.2 M [3-(N-morpholine) propanesulfonic acid]

10 mM EDTA, pH 7

50 mM sodium acetate

1% agarose-formaldehyde gel

0.5 g agarose

43.5 ml distilled water, melt together

5 ml 10X MOPS buffer

2.6 ml formaldehyde

20X SSC buffer

3 M NaCl

0.3 M trisodium citrate, pH 7

5X RPRB buffer

250 mM Tris-Cl, pH 8

25 mM MgCl₂

50 mM β-mercaptoethanol

100 μM each deoxyribonucleotide triphosphate (dNTP)

1 M HEPES, pH 6.6 with 4 M NaOH

SOLUTIONS FOR DNA GELS

50X Tris, acidic acid, EDTA (TAE) buffer

242 g Tris base

571 ml glacial acetic acid

100 ml 0.5 M EDTA, pH 8

make up to 1 L with distilled water

DNA loading buffer

30% glycerol

0.25% bromophenol blue in distilled water

0.8% Agarose gel

0.4 g agarose

50 ml 0.5 X TAE buffer

1 μl of ethidium bromide (10 mg/ml)

SOLUTIONS FOR CELL CULTURE

Sterile saline buffer

9 g/l NaCl, water, autoclave 20 min

Culture media

α-minimum essential medium (α-MEM)

2.2 g/L sodium bicarbonate

50 μg/ml gentamicin

0.25 μg/ml Fungizone

10% fetal bovine serum (FBS)

Pre-transfection media

α-minimum essential medium (α-MEM)

0.25 μg/ml Fungizone

10% FBS

Transfection media

Pre-transfection media minus the FBS

Selection media

Culture media with the addition of 50 µg/ml of ZeocinTM

Collection media

α-MEM supplemented with 50 µg/ml gentamicin

Appendix 2**A. Polyadenylated RNA isolation**

An affinity column was prepared by adding 0.5 ml of oligo (dT)-cellulose slurry suspended in loading buffer B (Appendix 1), into a sterile disposable plastic syringe packed with autoclaved glass wool. The column was washed with 3 ml of 0.1 M NaOH, 5 mM EDTA. The column was then washed with water until the effluent had a pH of less than 8. The column was subsequently washed with 5 ml of loading buffer A (Appendix 1) to equilibrate the column. The total RNA sample (10 mg) was dissolved in 500 µL of water, and secondary structure melted by incubation at 65°C for 5 minutes, then 500 µL of loading buffer A pre-warmed to 65°C was added. The sample was cooled to room temperature for 2 minutes then applied to the column. The elutant was collected and incubated at 65°C for 5 min, cooled to room temperature for 2 minutes, then reapplied to the column. The column was then washed with 5 ml of loading buffer B, this elutant contains non-polyadenylated RNA and was discarded. To the column 1.5 ml of elution buffer (Appendix 1) was added and polyadenylated RNA was collected, precipitated with sodium acetate and ethanol, resuspended in water and kept at -80°C until used.

B. Genomic DNA isolation from whole blood

The blood samples were thawed in ice water; 40 ml of triton/sucrose lysis buffer (Appendix 1) was added to 10 ml of blood and mixed by gentle inversion. Samples were centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the resulting white cell pellet was resuspended in 20 ml of triton/sucrose lysis buffer and then centrifuged at 3000 rpm for 10 minutes. Again the supernatant was discarded and the white cell pellet was gently resuspended in 3 ml of salting out/lysis buffer (Appendix 1), to this 200 μ l of 20% SDS and 75 μ l of 20 mg/ml (Fungal) Proteinase K (Gibco BRL) were added. Samples were incubated overnight with gentle agitation at 50°C. The next day 1.5 ml of 6 M NaCl was added to the sample and shaken vigorously for 15 seconds. The samples were then centrifuged at 3000 rpm for 15 minutes. The resulting supernatant was transferred to a new tube and 10 ml of absolute ethanol was added to precipitate the genomic DNA. The DNA strands were removed from the ethanol with a glass pipette and transferred to an eppendorf tube. The tube was centrifuged briefly to remove excess ethanol; the sample was resuspended overnight at 4°C in 400 μ l of Tris-EDTA (TE). The next day the samples were extracted once with phenol/chloroform, once with chloroform, then ethanol precipitated, rinsed with 70% ethanol, and allowed to resuspend overnight at 4°C in an appropriate volume of TE.

Appendix 3

C. Amino Acid Codes

| | | |
|---|-----|-----------------------------|
| A | Ala | Alanine |
| B | Asx | Aspartic acid or Asparagine |
| C | Cys | Cysteine |
| D | Asp | Aspartate |
| E | Glu | Glutamate |
| F | Phe | Phenylalanine |
| G | Gly | Glycine |
| H | His | Histidine |
| I | Ile | Isoleucine |
| K | Lys | Lysine |
| L | Leu | Leucine |
| M | Met | Methionine |
| N | Asn | Asparagine |
| P | Pro | Proline |
| Q | Gln | Glutamine |
| R | Arg | Arginine |
| S | Ser | Serine |
| T | Thr | Threonine |
| V | Val | Valine |
| W | Trp | Tryptophan |
| X | N/A | Unknown Amino acid |
| Y | Tyr | Tyrosine |
| Z | Glx | Glutamic acid or Glutamate |